Preclinical studies with Apoptin

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Contents:

In ruimten kruipen spinnen tussen buizen.
Ik wil het beest verslaan dat mij vergalt.

Tong slaat zich rauw op gril van elpen klippen.
Hoofd breekt zich over bron van lijfsbehoud.

Ik wil de pleisters van de goten rukken.
Het voorwerp dat mij stilt smeult in de hoek.

Blijmoedig wil ik wanhoop wijn inschenken.
Wat kan zal ooit en erger dan verwacht.

Ik wil ver heen mij nergens voort bewegen.
Ik zoek een bergzame plek om neer te zijgen.

Ik zal een feest begaan dat mij bevalt.

Uit: Nors en zonder haten. Piet Gerbrandy (1999).

Chapter 1

Introduction

Index

Introduction

Conventional cancer therapies, such as chemotherapy and radiation, target cell replication in general, damaging healthy proliferating tissues like the haematopoietic and gastrointestinal systems in the process. This activity in normal cells not only causes barely acceptable side-effects, it also limits the dose that can be administered, thereby hampering effectiveness. Two main issues have fostered the belief that therapies targeted specifically to tumour cell characteristics would be virtually impossible: (1) cancer cells do not develop entirely new traits, but deregulate cellular processes found in normal cells and (2) they do so by the acquisition of an indefinite variety of genetic mutations. Extensive research into the molecular changes of cancer cells, however, has recently led to the recognition that in order for a cell to become a tumour cell, e.g. to grow autonomously without responding to inhibitory mechanisms, a limited number of (incompletely) defined pathways is altered in every case. Therefore, the seemingly endless variety of mutations that occur can be grouped into this limited number of tumour essential pathways, with the biological endpoint of each altered pathway representing an indispensable trait of the tumourigenic phenotype. These new insights have opened the door for the development of novel therapies, termed molecular therapy of cancer, designed to target specific tumour characteristics, rather than targeting normal cellular processes or each and every individual mutation. To explain the molecular basis of tumour-specific therapies, this introductory chapter will first provide a summary of the hallmarks of cancer as they are currently defined and understood. Next, I will provide examples of how this knowledge can be translated into the development of novel therapeutic agents that cause tumour-selective cell death, based on already identified tumour essential pathways. Subsequently, some proteins are discussed that have shown tumour-selective cell killing but have not yet been linked to a distinct tumour trait. In addition to representing potential therapeutic agents by themselves, these proteins could confirm the described cancer hallmarks, identify new players in these pathways or even expose additional unrecognised tumour essential pathways, which in turn could lead to new cancer targets. One of the proteins in this category is Apoptin, and a summary of the current knowledge of its activity in tumour and normal cells will complete this chapter, preceding the first data on the activity of Apoptin *in vivo* presented in subsequent chapters.

From soldier to mutineer

Tumorigenesis is a multi-step process that reflects the acquisition of a variety of genetic changes in a somatic cell, each conferring one or another type of growth advantage. These traits are not consciously designed; rather, they arise under selection pressure. Hence, tumour development can be seen as analogous to Darwinian evolution; i.e. from a stochastic occurrence of genetic mutations, only those cells that have acquired an advantageous trait can continue to grow,

whereas all other cells with a less suitable mutation will perish or at best remain [1]. In fact, it has been an evolutionary imperative for multicellular organisms to efficiently suppress the genesis of mutated cells leading to deregulated growth, while at the same time allowing cell proliferation when needed [2]. Therefore, proliferation of individual cells in metazoans is highly regulated and subjected to a multitude of restrictions. These control mechanisms explain why cancer is a relatively rare event in an average human life time; many starting neoplasms fail to disable all restrictions and thus never reach the stage of true malignancy. Restrictions imposed on normal tissues include reliance on extracellular growth signals, requirement for survival signals generated by attachment to the extracellular matrix and soluble survival factors, the presence of growth inhibitory signals, a control mechanism in the form of programmed cell suicide and limited life span.

It has been known for some time that certain proteins are targeted frequently in all kinds of tumours arising under different circumstances. For instance, over half of all lung carcinomas were shown to have mutations in the tumour suppressor protein p53 [3], which also occurs in 30% of hepatocellular carcinomas [4], 65% of colon cancers [5] and ~ 40% of breast cancers [6]. Further, constitutive active forms of Ras [7], loss of heterozygosity of the RB locus [4] and activation of telomerase [8,9] occur frequently in human cancers. Tumours that do not have mutations in these 'hotspot' proteins are often found to have mutations in regulatory or effector proteins associated with them. In fact, studies in both mice and man have shown that mutations in hotspot proteins or their regulators are mutually exclusive indicating that a single pathway needs to be inactivated [10,11]. These data led to the notion that a limited number of key pathways exist that need to be affected in order for a cell to become cancerous. From the vast amount of research on molecular changes in cancer, a number of cancer 'hallmarks' have emerged and were enumerated by Hanahan and Weinberg [1]. Some represent the breaching of anticancer defence mechanisms, resulting in the ability to grow independently of growth signals, in the lack of growth control, resistance to apoptosis, and immortality. In addition, tumour cells will have to attract blood vessels and invade and metastasize into surrounding tissues. Finally, genomic instability enables tumour cells to 'stack the deck'; i.e. it provides the necessary platform for the frequent occurrence of *de novo* mutations. This set of acquired capabilities represents the fundamental difference between tumour and normal cells and provides crucial targets for cancer therapy. During the past decade, several of the underlying genetic changes that confer the essential traits to tumour cells have been characterized and will be summarized in the next chapter.

Multiple paths lead to cancer

The evolutionary aspect of carcinogenesis causes the particular sequence in which the tumour-essential capabilities arise to differ for each cell. Furthermore, the end result of the necessary feature can be achieved in numerous ways. Most, if not all, processes in a cell are regulated to allow the integration of different signals into the appropriate response. Alteration of a specific trait can therefore occur at different levels in such a signalling cascade. For instance, to acquire

continuous growth, a growth stimulating molecule can be amplified or its effector molecule might be mutated into a constitutively active form. On the other hand, an inhibitory molecule could be downregulated, mutated into an inactive form or completely abolished by deletion of some or all of its gene to obtain the same effect. Both quantitative and qualitative changes of positive or negative regulators, or effector molecules, can therefore result in the altered outcome of a particular signalling pathway. In addition, a specific genetic event could confer only part of a required trait to the cell, whereas another event could contribute to several tumour capabilities simultaneously. For instance, constitutive activation of a protein such as Ras that stimulates cell division but also facilitates cell movement contributes to both enhanced proliferation and invasion. The frequency with which a particular genetic event alters a pathway depends on the cell type and its associated selection pressure, giving rise to tissue-specific 'road-maps' to malignancy. Hence, certain tumour types will display mutations predominantly in a negative regulator of a pathway, whereas tumours arising from a distinct tissue acquire the same trait mainly by mutating the downstream effector.

Together, these features explain the seemingly endless diversity of genetic alterations observed in cancer. Nonetheless, all tumour cells are thought to reach the biological end-points described by the following hallmarks, which represent disruptions of common pathways (see figure 1).

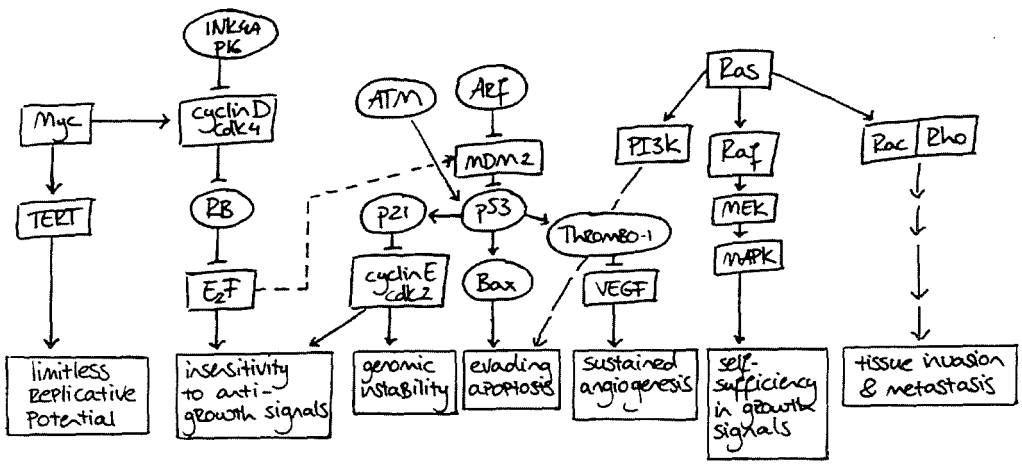

Figure 1 The hallmarks of cancer.

A new model of cancer has emerged postulating that most if not all cancers have acquired the same set of functional capabilities during their development. These characteristics are called 'cancer hallmarks' and are lined up at the bottom. Some key players in the processes conveying these capabilities are depicted; square boxes enclose proto-oncogenes and ovals, tumour-suppressor proteins. It is thought that perturbation of every one of these pathways is necessary to allow malignant growth, but different tumours have deregulated different proteins within a given cascade to achieve the same end result. For the purposes of clarity, this representation is simplified and illustrative rather than comprehensive. Loosely based on ref's 1 and 72.

Cancer Hallmarks

Growth stimulation

The decision to divide

Somatic cells are usually quiescent unless they are specifically instructed to proliferate. Growth factor receptors on the cell surface detect systemic (endocrine) signals or signals from neighbouring cells (paracrine). Most cell types are dependent on growth factors secreted by a different cell type in order to proliferate, thus preventing self-sustained proliferation. In addition, cells only respond to mitogenic signals that are tenacious; in other words, they need prolonged exposure for at least several hours before they enter the cell cycle [12]. In most tissues, mitogenic signals alone are not enough; a secondary signal is required to allow proliferation. This signal is conveyed by integrins that are connected to the extracellular matrix (ECM), thereby confirming that the cell is in its appropriate place. Successful binding to specific moieties of the ECM enables the integrin receptors to transmit survival signals into the cytoplasm that result in resistance to apoptosis and allow entrance into the active cell cycle [13]. The combination of dependency on growth factors secreted by surrounding cell types and the necessity to adhere to the ECM effectively 'traps' epithelial cells within a certain tissue that supplies their specified microenvironment [2]. Cells that are not physically connected to the ECM, like haematopoietic cells, face yet another restriction: antagonistic pleiotropy, in which a proliferative signal is usually accompanied by a death signal, and unless the latter is specifically overruled by additional survival factors, the cell will undergo programmed cell death instead of division [14].

Self-sufficiency in growth signals

To proliferate continuously, tumour cells need to become independent from endo- and paracrine growth stimuli, which can be achieved in several ways. In general, growth factors for a certain cell type are produced by a distinct cell type, one that is non-responsive to this particular factor. Some cancer cells are able to synthesize their own growth factors, such as platelet-derived growth factor (PDGF) or transforming growth factor-□ (TGF□), which in contrast to physiological heterotypic signalling, results in autocrine stimulation. Other cancers acquire a constitutively active component of the mitogenic cascade that results in ligand-independent signalling. Growth factor receptors usually have tyrosine kinase activities in their cytoplasmic domain which enable them to activate the Ras-Raf-Mitogen-activated protein (MAP) kinase cascade leading to mitosis. Mutant Ras proteins that provide continuous mitogenic stimulation are found in approximately 25% of all cancers [15]. These mutant forms have a constitutive GTPase activity leading to activation of the serine/threonine kinase Raf-1 which in turn phosphorylates MAPkinase kinase (MEK), which then phosphorylates extracellular-signal regulated kinase (ERK). At the end of this signalling cascade a number of genes driving proliferation are activated [16].

Growth control

Internal checkpoints

A web of growth inhibitory factors is superimposed upon the requirement for positive growth signals, and those must be overcome for cell-cycle entry. During the initial phase of the cell cycle, G1, cells acquire all the necessary information to proceed safely into the next phase, S, in which their genetic material has to be faithfully duplicated. Cells must not only evaluate their extracellular environment to ensure the presence of all necessary requirements (e.g. nutrient availability, mitogenic input, etc), but also must check whether there is any DNA damage that needs to be repaired before committing to cell division [17]. In early G1, removal of mitogens allows cells to return to G0. Later on in G1, however, the cells become committed to a new round of replication and are no longer dependent on mitogens. This point of no return is termed restriction point (R) and is regulated by the retinoblastoma (RB) family of proteins (figure 2). Phosphorylation of these proteins inactivates them and leads to progression of the cell cycle [18]. However, this decision to commit to a cell cycle is influenced by anti-proliferative signals such as those induced by transforming growth factor-β (TGF-β). TGF-β prevents phosphorylation of RB, thereby blocking a cell's advance through G1 [19]. Some cells, such as hepatocytes, are kept continuously in the quiescent (G0) state. However, after liver damage, these cells can be reactivated to enter the cell cycle in order to replace the lost hepatocytes. Alternatively, other cell types, thought to be at higher risk for mutations, are induced to relinquish their proliferative potential permanently by undergoing terminal differentiation.

Insensitivity to anti-growth signals

For progression through the cell cycle, two holoenzyme complexes must be formed to inactivate the key inhibitor of the restriction point, RB. These complexes consist of a cyclin associated with a cyclin-dependent kinase (Cdk), specifically, cyclin D1-Cdk4 and cyclin E-Cdk2. In quiescent cells, cyclin D1 levels are low, so cyclin D1-Cdk4 complexes do not form, and high levels of the Cdk-inhibitors p21 and/or p27 suppress cyclin E-Cdk2 activity (figure 2). Persistent mitogen stimulation induces cyclin D1 and regulates its assembly with Cdk4, a process facilitated by p21/p27 binding [20]. These Cdk-inhibitors are not very effective in blocking the cyclin D1-Cdk4 enzymatic activity and sequestration of p21/p27 by this complex results in the activation of cyclin E-Cdk2 as well [21]. Both G1 cyclin-dependent kinases can then collaborate to phosphorylate and inactivate RB and fellow family members p107 and p130. The repression of the E2F family of transcription factors by RB proteins is consequently released, allowing E2Fs to induce genes that are necessary for DNA replication [22,23].

Cyclin-D dependent kinases can be specifically inhibited by proteins from the INK4 locus. Whereas some of these latter proteins appear to be primarily involved in development, p16 plays a specialized role in countering signals that abnormally drive cell proliferation. Mice lacking p16 are tumour prone and develop a wide spectrum of cancers, particularly after exposure to

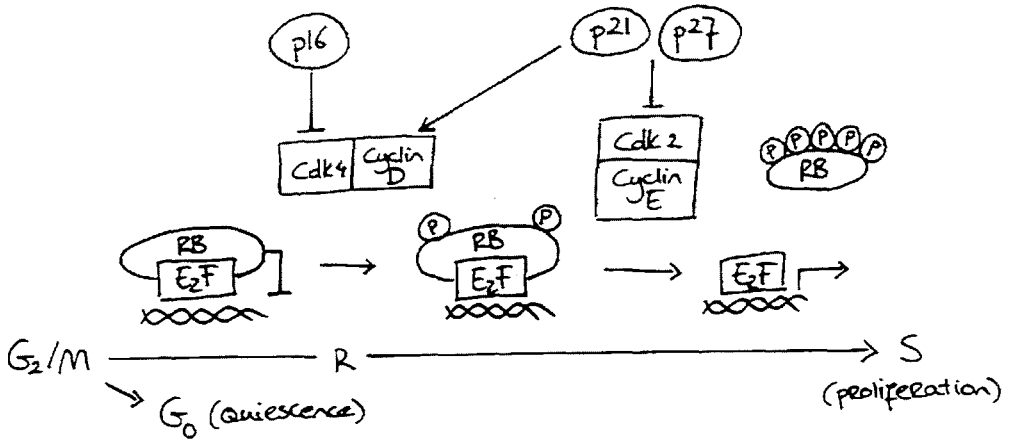

Figure 2. Regulation of G1/S transition by the RB pathway.

Progression through the G1 phase of the cell cycle is controlled by the functional state of the RB family of proteins, pRB, p107 and p130. In G_0, RB proteins are non-phosphorylated, a state that allows them to bind, among others, the E2F family of transcription factors, preventing E2F-dependent transcription. The cyclin-dependent kinases 4 and/or 6 become active upon binding to the mitogen-stimulated D-type cyclins. Phosphorylation of RB proteins by CDK4/6-cyclin D complexes leads to their partial inactivation, which allows transcription of E2F-controlled genes such as cyclin E1, which in turn activates the downstream CDK2 kinase. The activity of CDK4/6 is negatively regulated by the INK4 family of cell cycle inhibitors through prevention of binding to cyclin D. CDK4/6-cyclin D complexes may also activate CDK2 by binding p21/p27 proteins and thus preventing them from blocking CDK2-cyclin E activity. Hyperphosphorylation of RB proteins by CDK2-cyclin E complexes is required for proper G1/S transition and the initiation of S-phase. The position of the restriction (R) point is approximate. Adapted from ref. 18.

chemical carcinogens or X-rays [24-26]. During cellular senescence, p16 is selectively upregulated, as will be discussed with the cancer hallmark of achieving immortality (see section "Mortal cells").

Mutations in the pathway controlling G1 progression are found in virtually all tumours. RB disruption was first described in retinoblastoma, but is now catalogued in many other tumour types. Inactivation of p16 and overexpression of cyclin D1 or mutations in Cdk4 also occur frequently in cancer, and have been shown to be mutually exclusive with RB loss, providing evidence for both the necessity to inactivate this pathway and for the activity of these proteins within the same pathway. For example, in small cell lung cancer, 15% of the cases had inactivated p16, 5% overexpressed cyclin D or Cdk4 and 80% had lost RB [11]. The fact that the Cdk-inihibitors p21 and p27 are essential for cyclin D1-Cdk4 assembly renders their mutation unfavourable for tumour development and this indeed is less frequently observed [27,28].

Cell suicide

When in doubt: die

If any of the required signals to divide are not correct, the cell will undergo apoptosis, a multi-step cell-death programme that is present in latent form in every cell of the body. Because the subject of this thesis, Apoptin, exerts its anti-tumour activity by inducing apoptosis, I will describe this process and its regulation in more detail.

Apoptosis can be initiated either by extracellular death factors or by intracellular signals acting through the mitochondrial pathway (figure 3), both leading to the activation of cysteine aspartyl proteases (caspases). Many of the signals that elicit apoptosis following intracellular stress, such as genotoxic damage, lack of integrin signalling or oncogene activation, converge on the mitochondria, which respond by releasing cytochrome *c*. In the cytosol, cytochrome *c* forms a complex with Apaf-1 (apoptotic protease activating factor-1) and the inactive procaspase-9 [29]. Within this complex, known as the apoptosome, the initiator caspase 9 is activated and subsequently activates 'executioner' caspases, mainly caspases-3, -6 and -7. These active execution caspases cleave themselves to amplify the apoptotic signal, and also cleave an array of substrates resulting in the execution of the actual cell death, which is characterised by specific biochemical and morphological features [30]. For instance, the chromatin condenses due to disruption of lamins and the DNA is fragmented because the inhibitor of the endonuclease CAD (caspase-activated deoxyribonuclease) is cleaved and thus removed. In addition, caspase cleavage of cytoskeletal proteins such as actin leads to cell fragmentation and protuberances of the plasma membrane (blebs), ultimately giving rise to membrane-bound apoptotic bodies that contain the cell remnants. Exposure of phosphatidyl serines on these bodies facilitates engulfment and phagocytosis by neighbouring cells to prevent inflammatory reactions to contents that would otherwise be released from the dying cell [30,31]. Besides the prototypic caspase-dependent apoptosis, less-well-defined cell-death pathways that do not require caspase activation also exist. These pathways share some, but not all, characteristics of the process classically described as apoptosis [32,33].

Death receptor signalling is able to bypass the mitochondria and directly activate caspase-3 via caspase-8. When a death factor engages its cognate receptor, the cytoplasmic death domains recruit an intracellular adaptor protein, e.g. FADD (Fas-associated death domain protein), which in turn recruits and activates the initiator caspases-8 and -10 to form a death-inducing signalling complex (DISC) [34]. In some cell types, caspase-8 concomitantly induces cytochrome *c* release by cleavage of Bid, possibly to amplify the apoptotic signal in the case of naturally low levels of caspase-8 [35].

To allow sufficient control over tissue homeostasis, the apoptotic cascade is regulated at different levels. For instance, FADD-like adaptor proteins (FLIPs) can interfere with the binding of FADD to death receptors, thereby inhibiting caspase-8 activation [36]. In addition, Bcl-2 and several anti-apoptotic relatives act as guardians of mitochondrial integrity, among putative other

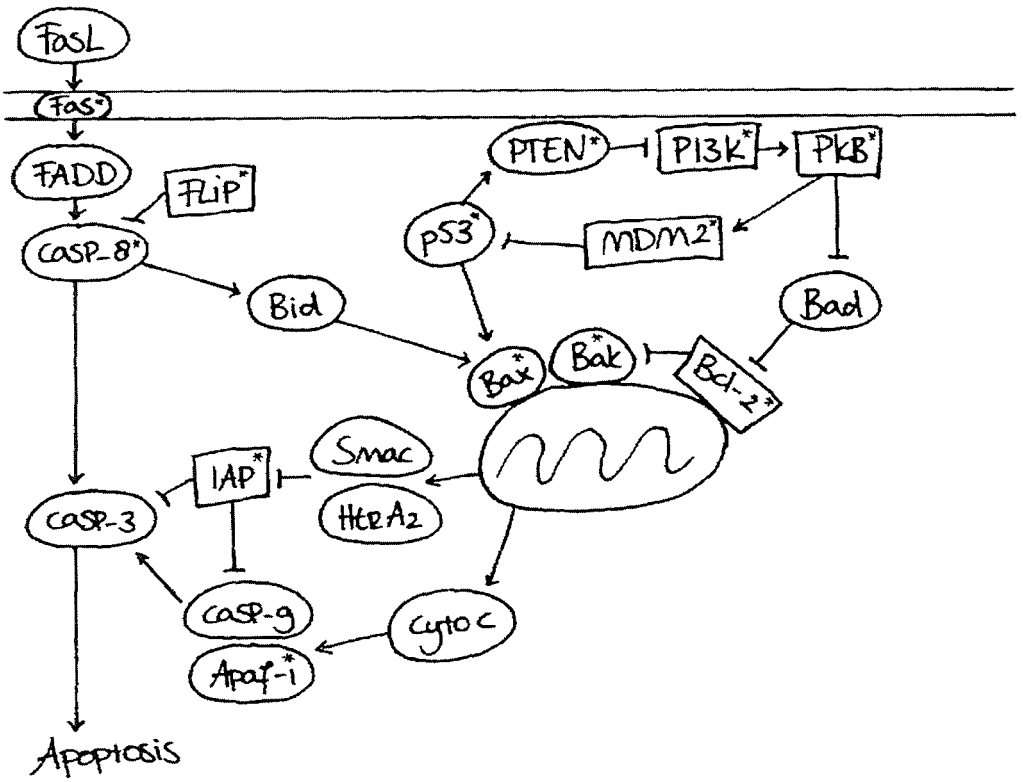

Figure 3. Apoptotic pathways.

A schematic diagram showing some of the known components of the intrinsic and death receptor apoptotic programmes. Proteins in squares inhibit apoptosis while those in circles promote apoptosis. See text for explanation. An asterisk denotes components that are frequently mutated or aberrantly expressed in human cancers. Abbreviations used: casp, caspase; cyt, cytochrome. Adapted from ref 244.

functions, and it seems that all mammalian cell types require the protection of at least one Bcl-2 homologue to ensure their existence [37]. Besides these pro-survival proteins, the Bcl-2 family contains two groups of pro-apoptotic proteins that share at least one conserved Bcl-2 homology (BH) domain: the Bax death family which contains three homology domains, and a more distantly related group, which shares only the short BH3 motif. The pro-apoptotic members stimulate cytochrome c release, whereas the anti-apoptotic members inhibit it, and the decision to undergo apoptosis is in part dependent on the balance between these proteins. For example, one means by which p53 elicits apoptosis in response to DNA damage is via upregulation of the expression of pro-apoptotic Bax [38].

In healthy cells, the BH3-only proteins are restrained in diverse ways, including phosphorylation and sequestration in the cytoskeleton. When released by death signals, BID-like

BH3 domains seem to activate apoptosis directly through targeting of Bax and Bak, whereas BAD-like BH3 domains 'sensitise' or enable apoptosis by neutralizing Bcl-2 and related pro-survival proteins [39]. BH3-only proteins cannot kill in the absence of Bax and Bak, which apparently act further downstream. Bax and Bak oligomerise in the mitochondrial outer membrane and probably breach its integrity, releasing pro-apoptotic proteins such as cytochrome *c*. However, the precise mechanism by which Bax and Bak are activated, and how they are inhibited by Bcl-2 remain uncertain. In addition, it has become clear that Bcl-2 can also protect against certain types of stress-induced apoptosis, such as that initiated by calcium-deregulation in the endoplasmatic reticulum [40]. Under those circumstances, mitochondrial disruption is neither necessary nor sufficient, indicating that Bcl-2 might prevent the activation of caspases independently from its function at the mitochondria [37].

Downstream of the mitochondria, apoptosis is modulated further by inhibitor of apoptosis proteins (IAPs) that bind to and inhibit activated caspases, in some cases by targeting them for degradation [41]. However, when mitochondria are breached, they release a series of proteins that promote cell death. Besides cytochrome *c*, these include SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI) and the serine protease HtrA2/Omi, both of which antagonize IAPs. Also the apoptosis-inducing factor AIF and endonuclease G are released and facilitate DNA fragmentation, thereby adding weight to the pro-apoptotic side of the balance [37].

The high degree of regulation surrounding the apoptotic process ensures that cells are able to integrate different survival and death signals to come to the appropriate decision. In some cells, such as those of haematopoietic origin, apoptosis is the default status that needs to be specifically overruled by survival signals. Ligation of interleukin (IL)-3 with its cognate receptor on haematopoietic cells results in the activation of the phosphatidylinositol-3 kinase (PI3K), which ultimately causes phosphorylation and thereby inhibition of pro-apoptotic BAD and FOXO3a [42,43]. Survival factors, however, are often limited in supply and spatially restricted, preventing continuous growth [44,45]. In the case of epithelial cells, the disruption of contact with the extracellular matrix results in loss of survival signalling via PI3K and thereby cell death, in this case termed anoikis.

An important regulator in the decision to die upon intracellular stresses such as DNA damage, hypoxia or shortened telomeres (see section "Mortal cells") is the tumour suppressor protein p53. Dependent on the cell type, cell environment, strength of the insult and intracellular p53 levels, the cellular response to p53 activation can be DNA repair, differentiation, cell-cycle arrest, apoptosis or senescence. P53 can induce expression of several death effectors in both the death-receptor and mitochondrial apoptotic pathways, such as Fas, Bax and the BH3-only proteins Noxa and Puma. In addition to inducing these pro-apoptotic genes, p53 can also activate the expression of genes that inhibit survival signalling [46]. For instance, PI3K is an activator of protein kinase B (PKB, a.k.a. Akt) that phosphorylates and activates MDM2, which in turn targets p53 for degradation. The inhibition of p53 by PKB, however, is counteracted by the ability of p53 to induce expression of PTEN, a phosphatase that can inhibit the activation of

PKB [47] [48]. Moreover, the p53 pathway is connected to the RB pathway in that deregulated E2F activity induces the transcription of ARF, which is an alternative reading frame of the INK4a locus encoding p16. ARF negatively regulates MDM2, thereby increasing p53 stability [49]. Thus, p53 is a crucial sentinel integrating the response of a cell to survival, proliferation and damage signals, and the various pathways are intimately interconnected.

Evading the death sentence

In general, any of the cellular responses to p53 activation, be it cell cycle arrest, senescence or apoptosis, inhibits tumour development [48]. Moreover, because disruption of the RB pathway triggers compensatory p53-dependent cell cycle arrest or even apoptosis, dysregulation of the cell cycle (the above-mentioned hallmark) results in an additional imperative for tumour cells to disrupt the p53 pathway. Malignant progression is therefore dependent on loss of p53 function, either through mutation in the p53 gene itself or by defects in the signalling pathways that are upstream or downstream. The importance of inactivation of the p53 pathway either directly or indirectly is underscored by the finding that any impairment of p53 function is associated with increased tumour susceptibility in mice [50], together with the fact that mutations in p53 are found in about half of all human cancers [51]. Alternatively, many cancers show enhanced activity of the PI3K/PKB signalling cascade to avoid apoptosis, either by loss of PTEN or through amplification of the catalytic subunit of PI3K, depending on the tissue of origin [52].

Besides altering the p53 and PI3K pathway, tumour cells can acquire resistance to apoptosis by overexpression of anti-apoptotic molecules or by inactivation of pro-apoptotic genes. In fact, the first clue that apoptosis was important for cancer was the discovery that translocation of the gene BCL-2 (B-cell lymphoma-2) in follicular lymphoma resulted in reduced cell death rather than in enhanced proliferation [53]. Later on, mouse studies confirmed the oncogenic potential of elevated Bcl-2, although the stochastic onset of tumours in mice mimicking the BCL2-translocation indicated that additional mutations were required for transformation [54]. Particularly, dramatic synergy was observed when Bcl-2 was co-expressed with oncogenes that enforce proliferation, such as Myc [55,56]. As the Bcl-2 pro-survival proteins are all likely to be oncogenes, the pro-apoptotic members probably constitute tumour suppressors and thus need to be inactivated during carcinogenesis. Specifically, downregulation of Bax is commonly found in haematopoietic malignancies [57] and absence of both Bax and Bak enhance transformation far greater than the loss of either alone [58]. Further, metastatic melanomas often lose expression of Apaf-1 [59], whereas apoptosis through death receptors is averted by overexpression of FLIP, simultaneously allowing the tumour cell to escape T-cell mediated cytotoxicity [60].

Notwithstanding the well-documented necessity for tumour cells to inactivate apoptotic signals, many tumours *in vivo* exhibit a high percentage of apoptotic cells. This state is a consequence of the evolutionary nature of tumorigenesis, in which mutations are selected by environmental pressure (figure 4). Premalignant cells that completely lack inhibition of apoptosis are obviously unable to establish an autonomously growing colony. However, premalignant cells do not get to 'choose' the alteration with the most profound effect; rather, they stochastically

acquire mutations. If this mutation allows the cell and its progeny to undergo more cell divisions before it is eliminated by apoptosis, a tumour mass will arise. Consequently, distinct tumours can display substantial differences in their ability to evade apoptosis; some may be endowed with a robust block downstream in the apoptotic cascade, whereas in others, only some of the key initiating factors of apoptosis have been disabled, resulting in delayed apoptosis via other signals, nevertheless cumulating in a selective growth advantage.

Mortal cells

Live short and prosper

The finite life span of mammalian cells forms another barrier to tumorigenesis. When cells are explanted from living tissues into culture, they undergo only a limited number of cell divisions before arresting in a state known as replicative senescence. Such cells are irreversibly arrested in the G1 phase of the cell cycle and are no longer sensitive to growth factor stimulation. When an oncogene such as Ras becomes overactive, cells will undergo the same fate, in this case termed premature senescence. There has been some debate whether senescence is related to *in vitro* cell culture rather than to a relevant mechanism occurring *in vivo*. Recently, however, it was demonstrated that p53 and p16 cooperatively induce a senescence programme *in vivo* [61]. In these studies, murine lymphoma harboring mutated p53 responded poorly to chemotherapy due to their apoptosis defect. But when the apoptotic block was downstream of p53, these lymphomas underwent a drug-induced senescence programme that was dependent on intact p53 and p16, significantly increasing the prognosis of these mice compared to those sustaining lymphoma due to direct p53 mutations. Concordantly, to overcome the senescent state *in vitro*, cells need to inactivate both RB and p53 [62]. The cells are then able to continue growing, but only to a certain point termed crisis. Crisis is characterized by massive cell death and karyotypic disarray associated with end-to-end fusion of chromosomes [63,64]. The limited number of cell doublings endowed to each cell is thought to be calculated by progressive telomere shortening. Telomeres cap the ends of eukaryotic chromosomes with tandem repeats of the sequence TTAGGG bound to a complex array of proteins, preventing the ends of the chromosomes from being recognized as DNA breaks, and, therefore, from being processed by DNA-repair activities. With each round of cell division, the 3' ends of chromosomal DNA are incompletely replicated, leading to the progressive shortening of the telomere. Critically short telomeres are detected as damaged DNA and result in activation of the DNA-damage checkpoint, leading to cell-cycle arrest and/or apoptosis [65,66].

Whereas telomere shortening occurs in most somatic cells, certain cell types, such as germ cells and a subpopulation of stem cells, have an active enzyme-complex that prevents net telomere shortening as they divide. This enzyme, called telomerase, is a cellular reverse transcriptase that comprises a catalytic subunit, TERT, and an RNA component, TERC, and exerts its function by adding TTAGGG repeats onto pre-existing telomeres [65]. In this way,

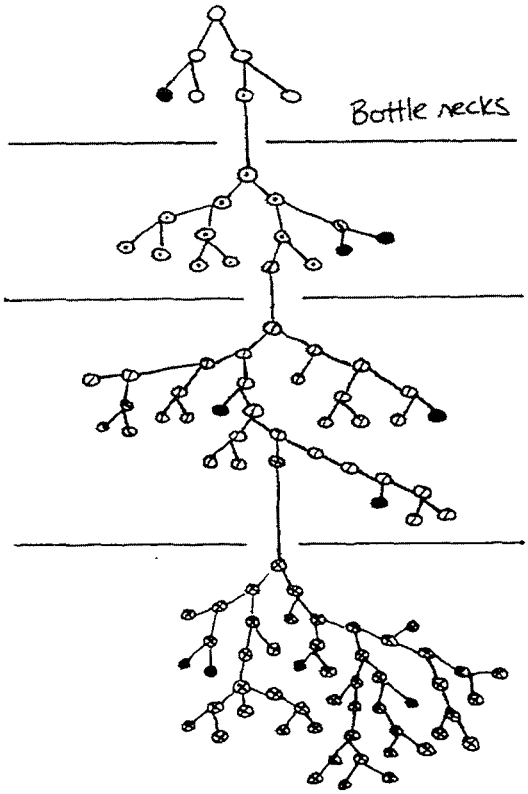

Figure 4. Clonal evolution of a cancer

All cancers evolve by Darwinian principles: clonal proliferation, genetic diversification within the clone, and selective pressure enabling mutant subclones to bridge the bottlenecks (such as cell-cycle checkpoints and apoptosis imposition). This simplified diagram illustrates the large number of cell divisions required to obtain all functional mutations for the cancer-selective capabilities, as well as the simultaneous existence of an apoptotic population within the growing colony. Each circle represents a cell, acquiring the first (dot) or additional, sequential mutations (/ and x). Black circles represent dying cells. Adapted from ref 245.

telomerase maintains telomeres above a minimum-length threshold, thus preventing telomere exhaustion and chromosomal instability and allowing immortal growth.

Limitless replicative potential

As discussed above, tumour masses arise when their proliferative potential exceeds their apoptotic onslaught, a requirement that partly explains why the growth of tumours *in vivo* is relatively slow. Suppose, for instance, that of every nine new tumour cells, six die rapidly. In this case, the mass will increase at only one third of the rate of its proliferation. Therefore, an enormous amount of cell doublings are required to form a tumour [67] (figure 4). As the tumour cells continue to proliferate, they will eventually reach the natural end of their life span, indicated by critically short telomeres. These dysfunctional telomeres signal DNA damage repair and subsequently cell-cycle arrest and/or apoptosis. The latter need to be overcome in order for malignant growth to continue; this usually occurs by reactivation of telomerase. But by this point, the dysfunctional telomeres will have been subjected to DNA repair, resulting in end-to-end chromosomal fusions and the occurrence of anaphase bridges during mitosis, which could contribute to the genetic instability that is characteristic of cancer cells.

The importance of disabling this life span 'clock' is demonstrated by the finding that over 90% of all human tumours have reactivated telomerase at some point during their development to achieve immortality [68-70]. In addition to its telomere-preserving activity, telomerase has recently been suggested to have additional roles in tumour growth and survival by accelerating the growth of epithelial cells through regulation of genes that mediate the RB-E2F pathway [71].

Of note, human cells need to circumvent two barriers, cellular crisis and replicative senescence, to achieve immortalisation [72]. Mouse cells, on the other hand, have telomeres 3-10 times longer than do human cells and express constitutive telomerase in most tissues. Telomeres, therefore, do not seem to limit the replicative lifespan of mouse cells. In addition, even though the study from Schmitt et al. implicated p16 in the replicative senescence of murine cells [61], other studies have demonstrated a dominant role for the p53 pathway in mouse cells. Specifically, ablation of either Arf or p53 seemed sufficient to overcome Ras-induced senescence in mouse cells *in vitro*, in contrast to human cells that needed to inactivate both the RB and p53 tumour-suppressor pathways [72]. These differences may partly explain why it is so difficult to transform human cells, whereas rodent cells can undergo spontaneous immortalisation following extended *in vitro* culture [73]. Hence, although mouse models can and have provided valuable insights into the biology of human cancer, it is important to keep in mind that fewer cancer hallmark pathways are likely to be involved in murine carcinogenesis than in human.

Blood supply

Formation of blood vessels

During embryogenesis and in wound healing and inflammation, new blood vessels are induced to extrude from existing vessels, a process called angiogenesis. Angiogenesis can be initiated by local hypoxia, a process that activates hypoxia-inducible factor-1α (HIF-1α), which in turn promotes the expression of several growth factors responsible for endothelial recruitment and proliferation [74,75]. The initiation of angiogenesis is regulated by a balance of angiogenesis inducers and inhibitors. Well known stimulators of angiogenesis include vascular endothelial growth factors (VEGFs), which act through a family of tyrosine kinase receptors, VEGFRs [76]. A prototypical angiogenesis inhibitor is thrombospondin-1, which binds a transmembrane receptor on endothelial cells coupled to intracellular Src-like tyrosine kinases [77].

Sustained angiogenesis

Tumours cannot grow beyond a certain size without sufficient blood to supply oxygen and nutrients and therefore must induce angiogenesis when the tumour mass exceeds ~ 1 mm^3 [78]. In some tumours, thrombospondin itself is downregulated [79,80], but thrombospondin is also a downstream effector of p53 [81]. Consequently, loss of p53 results in a drop of thrombospondin-1 levels as well, shifting the balance towards angiogenesis. Alternatively, cancer cells start producing angiogenic factors including basic-fibroblast-like growth factor (bFGF), vascular

endothelial growth factor (VEGF), interleukin 8 (IL-8) and transforming growth factor-β (TGF-β). Several cancer types exhibit overexpression of VEGF, but new insights suggest that surrounding 'normal' cells can also play a role in tumorigenesis, for instance by upregulation of VEGFR in tumour-surrounding endothelial cells [82].

Tissue remodelling

Cellular socialization

Cell-cell and cell-matrix contacts are important in inhibiting inappropriate growth and are thus obligatory for a cell to ensure its survival and proliferation. Complementary to the activation of PI3K signalling by integrin-ECM attachment, cell-cell connections endorse proliferation via the Wnt signalling pathway. Cell-cell adherens junctions assemble by means of interactions among the extracellular domains of cadherin receptors on the surface of adjacent cells. The cytoplasmic domains of cadherins bind to β-catenin or plakoglobin (γ-catenin), which are linked to the cortical actin cytoskeleton by α-catenin [83]. Most of the β-catenin present in the cell is sequestered by E-cadherin. Free β-catenin is rapidly phosphorylated by glycogen kinase 3β (GSK3-β) in the adenomatous polyposis coli (APC)/GSK3-β/axin complex and subsequently degraded by the ubiquitin-proteasome pathway. When GSK3-β activity is blocked by activation of Wnt signalling after ligation to its cognate receptor, β-catenin accumulates at high levels in the cytoplasm. Subsequently, it translocates to the nucleus, where it binds to members of the T cell factor/lymphocyte enhancer factor (TCF/LEF) family of transcription factors, whose targets include c-Myc, cyclin D1 and metalloproteinase matrilysin (MMP-7) [84,85].

The body also requires non-resident cells, not only for the delivery of oxygen and nutrients, but also for immune surveillance. In order to patrol through different tissues in search for pathogens, immune cells need a complicated set of capabilities including the ability to 'extravasate' from blood vessels into a tissue, pass through the basal membrane and move from one tissue to the next. Extravasation requires adhesion and disassociation with endothelial ligands while migrating through the endothelial layer, a process termed diapedesis [86]. Next, the basal membrane, consisting of collagen, laminin and proteoglycans, needs to be degraded by the activation of matrix metalloproteases (MMPs) [87,88]. To achieve mobility, several intracellular pathways must be activated, including Ras/MAPK and the tyrosine kinase receptor c-*met*, responsible for cytoskeletal reorganizations and the induction of lamellipodia, extrusions from the cytoplasm that provide anchorage and direction [89,90].

Invasion & metastasis

In order for tumours to metastasise and grow, neoplastic cells must invade and migrate into surrounding tissues. These phenotypic changes are initially mediated by alterations in the expression of cell-surface molecules such as integrins, release of proteases that remodel the ECM, and the deposition of new ECM molecules. These activate signalling cascades that regulate gene expression, cytoskeletal reorganization, cell adhesion and cell survival. These

alterations then contribute to enhanced migration and proliferation, leading to invasion, metastasis and an improved ability to survive in different microenvironments [91].

The importance of cell-cell contact is underscored by the observation that hereditary predisposition to gastric cancer results from germline mutations in the E-cadherin gene CDH1 [92,93]. Furthermore, loss of E-cadherin is associated with the transition from adenoma to carcinoma and the acquisition of metastatic capacity [94]. Mutations in the tumour-suppressor gene APC are frequently detected in colon cancer. Importantly, disruption of cell-cell contact not only decreases cellular adhesion, but it also results in altered intracellular signalling. The combination of loss of cell adhesion with activation of β-catenin-mediated transcription might explain why mutations in this pathway are found in cancer, although they seem mostly restricted to gastrointestinal varieties [85].

Besides facilitating escape to and from the circulation by degrading the basal membrane, matrix metalloproteases (MMPs) have a significant effect on the ability of tumour cells to grow in a secondary site [95]. Both upregulation of MMP expression or downregulation of endogenous MMP inhibitors such as TIMP1 can be observed in human cancer [96].

The finding that genetic analyses of biopsies from breast carcinoma *in situ* (i.e. restricted to one site) can already predict whether this tumour will eventually metastasise, has prompted some to state that 'invasion and metastasis' does not represent a cancer hallmark but merely emerges (or not) from the other hallmarks [97,98]. However, it could also be that, since the metastasis phenotype actually encompasses a whole set of acquired capabilities, this hallmark is dependent on two distinct determining factors. It is probable that part of the capabilities for invasion are provided by other pathways, such as Ras activation, which also influences cytoskeleton mobility, but other capabilities so far appear to be unrelated to the remaining pathways, such as MMP expression. If the stochastic selected mutations in the hallmark pathways described previously represent the most favourable setting for a tumour cell to invade, then additional capabilities are likely still needed to degrade the basal membrane, intra- and extravasate and home in a new tissue. In this setting, such capabilities could be attained by just a single mutation in e.g. an MMP-1 regulator. But when the necessary alterations in the pathways of the other hallmarks have taken place at an unfavourable level, for instance by activation of a downstream activator of Ras instead of Ras itself, this would amount to a whole set of additional mutations that must be acquired by this tumour to make invasion possible. The chance of acquiring all these new mutations versus the acquisition of only a few could be the determining factor that predicts whether this particular tumour will be able to metastasize. It could even be that certain mutations in other hallmark pathways are incompatible with invasion. In any case further research is needed to establish exclusive alterations for invasion/metastasis that cannot be achieved by alterations in the other hallmark pathways, which would then validate the existence of the cancer hallmark, 'invasion and metastasis'.

Genetic instability

Preventing the fixation of mutations

Endogenous DNA damage occurs at a high frequency, whether base loss by spontaneous hydrolysis of DNA glycosyl bonds [99], pyrimidine dimerization elicited by UV light [100], or breakage of a double-stranded DNA molecule due to ionizing radiation [101]. As many mutations result in loss of function or even lethality, repair of these lesions is crucial to a cell's survival. It is also important to finish the repair before a cell enters S phase and the mutations become 'fixed', i.e. passed on to a daughter cell unable to recognise nor repair the mutation. Different types of DNA lesions are recognised and repaired by different sets of proteins; for instance, bulky DNA adducts such as UV photoproducts are resolved by nucleotide excision repair (NER), and double stranded breaks by homologous recombination or non-homologous endjoining. Usually, DNA damage sensors continuously 'scan' the DNA for aberrancies and subsequently recruit the repair machinery in a local fashion, ensuring that the damage is repaired quickly. However, if the repair is slow due to multiple lesions at one site or genomic localisation, a global DNA damage response is triggered (also termed DNA damage checkpoint), by activating a signalling cascade involving Rad53p and Chk1p that arrests cell-cycle progression until repair has occurred [102,103].

Stacking the deck

The concept that the genesis and progression of cancers are based on natural selection of stochastically acquired mutations implies that *de novo* mutations need to occur frequently. This process might be accelerated by loss of DNA repair functions and checkpoints, for instance by p53 mutation, but also other genomic caretaker proteins are found to be lost in cancer [104]. Indeed, most cancers exhibit not only subtle genetic alterations but also dramatic karyotypic changes, including gain and loss of chromosomes, gross chromosomal rearrangements, and amplification or deletion of DNA segments. However, the extent to which genomic instability is a prerequisite for tumour development remains unclear, as it could be a mere consequence of a tumourigenic event such as telomere erosion. And although many tumours show genomic instability, others are found to have relatively stable genomes [2]. Therefore, an intense debate is currently ongoing about the role of genetic instability in cancer [105].

Some state that a mutator phenotype is required to obtain all necessary mutations conveying the former cancer hallmarks within a human life time [106]. The difficulty in determining the mutation rate in normal cells coupled with new insights into the number of cell divisions necessary to reach a certain tumour mass (see section on limitless replicative potential) have prompted others to favour the idea that normal mutation rates would be sufficient to transform a normal cell into a malignant one [67]. This idea is supported by studies that fail to detect gross chromosomal abnormalities in premalignant human cancer [107], suggesting that genomic instability is not involved in the initiation of cancer.

Convincing evidence for the opposite view, however, comes from studies by Lengauer and Vogelstein [108-111]. They compared the accumulation of gross chromosomal changes in two types

of colon cancer cells: those with a mismatch repair defect and those without. Cancer cells with a mismatch repair defect contained a normal chromosome number even after the course of many cell divisions. In contrast, cells that had become cancerous due to other mutations acquired chromosomal changes 10 to 100 times faster than the mismatch repair defective cells. If genomic instability would have been a consequence of malignancy, the mismatch repair deficient cells should also have displayed chromosomal disarray over time. Moreover, this group did detect major chromosome abnormalities in biopsies of very small precancerous colon adenomas. These data support that an increased rate of mutation, achieved by either the absence of DNA repair or by cruder measures, is required for the ability of a cell to become a tumour cell, rendering genomic instability a cancer hallmark.

Reconstituting cancer

To provide further evidence that the genetic alterations associated with the hallmarks of cancer are indeed necessary and sufficient to transform normal cells into tumour cells, Hahn and coworkers performed a series of elegant reconstitution experiments [112]. The minimal required elements to enforce tumourigenic conversion of normal human cells proved to be the ectopic expression of the telomerase catalytic subunit in combination with an oncogenic allele of H-*ras* and the simian virus 40 (SV40) early region. Not only were these cells no longer dependent on adhesion for their growth and had gained an infinite life-span, this combination of genetic changes also resulted in *in vivo* tumour formation in nude mice including an invasive phenotype [113]. The SV40 early region encodes at least two proteins: the large T (LT) and small t (st) antigens. The LT oncogene is known to inactivate both p53 and RB, but it also has additional functions whose significance are largely unknown [114]. Replacement of LT by two other viral oncogenes known to inhibit p53 and RB, human papilloma virus proteins E6 and E7, respectively, was not sufficient to convey tumourigenic growth to primary cells in the presence of active Ras and telomerase activity. Addition of st antigen on the other hand was again able to complement Ras, telomerase and the disruption of p53 and RB, in inducing the oncogenic phenotype, indicating that at least one other pathway is perturbed by both LT and st that is required for tumorigenesis [112]. Although the myriad functions of these viral proteins makes it difficult to determine the exact pathways involved, these studies nevertheless indicate that perturbation of a limited number of pathways is sufficient to transform normal cells into fully malignant cells. They also convincingly showed that it is unlikely that the reconstituted tumour cells had acquired additional mutations in their culture time prior to the injection into nude mice, since there were no signs of genetic instability [115]. This does not argue against the view that genomic instability is a cancer hallmark, because in this case, all mutations have been directly introduced, in contrast to the selection process in regular carcinogenesis where only occasionally a necessary mutation arises amidst thousands of irrelevant mutations. Of note, these studies have been conducted with human cells in immune-compromised mice, and therefore preclude any comments on the effect of the immune system on tumorigenesis.

Evidence exists that under some conditions, immunosurveillance plays a role in eliminating tumour cells, consequently adding an additional selection force to the arising tumour mass. In other words, only those tumour cells that have acquired mutations preventing immune attack will continue to proliferate. The current debate over whether immune escape is indeed a cancer hallmark is summarized in references 116 and 117. In addition, the reconstitution studies have focused on adherent cells and it can be envisaged that the acquisition and requirement of cancer hallmarks that apply to non-adherent cells, e.g. from the haematopoietic system, differ.

Not only does a limited number of cancer-hallmark pathways seem to suffice for tumorigenesis, but their disruption appears to be required throughout tumour progression to maintain the malignant phenotype, despite numerous additional genetic alterations. For instance, stable inactivation of oncogenic alleles of K-*ras* in human pancreatic carcinoma cells through use of a viral RNA interference vector led to loss of tumourigenicity (as measured by soft agar assays and growth in nude mice) [118]. In addition, re-expression of wild-type p53 in tumour cells with mutant p53 results in apoptosis, regardless of multiple genetic changes [119,120]. Further, full-blown pancreatic tumours in transgenic mice caused by an inducible *myc* ongene completely disappeared after removal of ectopic myc expression [121]. These studies reveal that tumour cells remain dependent on their malignant alterations; a fact that may provide an opportunity for therapeutic approaches targeting tumour cells specifically while leaving normal cells unharmed.

Molecular therapy of cancer

Although the differences between tumour and normal cells appear subtle, based as they are on the deregulation of normal cellular processes, significant qualitative and quantitative differences have now been identified. Not only do tumour cells remain critically dependent on the acquired mutations, but they also lose flexibility in responding to altered circumstances and cytotoxic insults. Due to continuous activation, overexpression or complete ablation of certain factors, responses can no longer be accurately modified. This inflexibility most likely contributes to the therapeutic window achieved with chemotherapy and radiation. Notwithstanding the dramatic improvement in survival of cancer patients since the implementation of these drugs, new therapies are still required to be able to successfully treat all cancer patients. Besides their dose-limiting toxicity, which prevents total eradication of certain tumours, chemo- and radiation therapy mainly depend on intact apoptotic pathways to exert their anticancer activity. Because these same pathways need to be inactivated in one way or another to allow malignant progression in the first place, many tumours are rendered resistant to this type of therapy. Studying the molecular basis of cancer, resulting in the recognition of mandatory alterations in a restricted number of key pathways for the establishment of all tumours, has allowed the rational design of cancer therapies that should be more refined and more effective. Succesful examples of such molecular therapeutic approaches will be described in the following paragraphs. In addition, the identification of proteins that selectively kill tumour cells can contribute to

understand carcinogenesis further, and in turn provide an opportunity for the development of novel therapeutic strategies, as will be exemplified in the second half of this chapter.

Tumour-specific therapies

Tyrosine kinase inhibitors

The drug trastuzumab (Herceptin) serves as the first success story for therapeutic strategies based on molecular studies of oncogenes. An accidentally cloned gene encoding a growth factor receptor for endothelial growth factor (EGF), termed HER2 (human EGFR-related gene), resulted in the discovery that the gene encoding the HER2/neu receptor tyrosine kinase is amplified up to 100-fold in the tumour cells of about 30% of patients with invasive breast cancer [122]. Genentech Inc. developed an antibody directed to the extracellular domain of HER-2, called trastuzumab, which showed good antitumour activity in preclinical models, particularly when used in combination with a second agent such as doxorubicin or paclitaxel (Taxol) [123]. The therapeutic antibody appears to work by several mechanisms including internalization and down-regulation of HER-2 receptor, induction of cell-cycle inhibitors such as p27 and immune-mediated responses [124]. Trastuzumab is well tolerated by patients and the drug was approved by the U.S. Food and Drug Administration (FDA) in 1999 for the treatment of HER-2-positive breast cancer [125]. Responses of up to 60 to 70% have been noted for trastuzumab in combination with paclitaxel. Improved efficacy was also achieved in patients treated with both trastuzumab and doxorubicin, but this combination also results in greater cardio-toxicity [126], likely because HER-2 is expressed in heart tissue and doxorubicin is known to be a cardio-toxic agent [127].

Another tumour-specific elevation of tyrosine-kinase activity was discovered in chronic myelogenous leukaemia (CML) resulting from a chromosomal translocation, known as the Philadelphia chromosome, that creates a fusion of the Bcr and Abl genes. A pharmacological screen aimed at finding a selective inhibitor of this tyrosine kinase yielded a phenylamino derivative that was further refined into the compound called Glivec (or Gleevec or imitanib) [128]. An *in vitro* screen identified two other kinases effectively inhibited by Glivec, namely c-KIT and PDGFR. Glivec was shown to prevent downregulation of the cell-cycle inhibitor p27 by *BCR-ABL* and to induce apoptosis in cells from CML patients by suppressing the activation of the antiapoptotic Bcl-x_L protein [129,130]. After corroboration of preclinical data with impressive responses in clinical trials for CML, Glivec was approved by the FDA in 2001 [131]. The activity of Glivec against c-KIT, eliminating its constitutive MAPK and PKB activation, and against PDGFR, inhibiting autocrine growth factor stimulation by tumourigenic PDGF expression, is being evaluated for the treatment of other tumour types such as gliomas and ovarian cancer [128]. Two other inhibitors of a distinct tyrosine kinase –the epidermal-growth-factor-receptor (EGFR)- called Tarceva and Iressa, are currently in phase III clinical trials [127,132].

Farnesyltransferase inihibitors

Activating oncogenic mutations of the *ras* gene are common in cancer, occurring in 30% of solid tumours in adults. Inhibitors of the enzyme farnesyl protein transferase prevent an essential lipidation of the Ras oncogene proteins; without this lipidation, Ras signalling is inhibited. However, at least 20 other proteins have been identified so far which are also substrates for farnesyl transferase, including RhoB and downstream effectors of PI3K, thus farnesyltransferase inhibitors (FTIs) cannot be considered Ras-specific drugs [127,133,134]. Whether inhibiting the farnesylation of other proteins is involved in mediating the transformation-selective properties of FTIs has yet to be established. Paradoxically, FTIs had little effect on the proliferation of untransformed cells, although the function of wildtype, farnesylated Ras protein is important for their proliferation [134]. In patients, general farnesyltransferase inhibitors showed some activity against gliomas but also caused substantial toxicity [7]. Specific inhibitors of FT have yielded more encouraging results in phase I and II clinical trials with up to 30% response rates in haematologic malignancies trials. Although the clinical effects are more modest than was expected based on the preclinical data, the toxicity is manageable, and several FTIs are currently undergoing further clinical testing in combination with chemotherapeutics and radiation [135,136].

Targeting apoptosis pathways

Unfortunately, one of the requirements for cancerous growth, namely inactivation of apoptotic pathways, concomitantly limits the effectiveness of therapies that exert their anticancer activity via the induction of apoptosis. Particularly, specific mutations in p53 have been linked to primary resistance to doxorubicin treatment and early relapse in patients with breast cancer [137]. Reduced Bax expression was also associated with a poor response to chemotherapy [138]. Furthermore, it has been shown that Bcl-2 expression confers resistance to many kinds of chemotherapeutic drugs and irradiation [139-141].

On the other hand, the blockage of apoptosis in tumour cells is not necessarily robust. Many tumour cells barely survive and they are highly sensitized to apoptosis due to a barrage of death signals that must be continuously overruled [2]. The consequence of an apoptotic signal might therefore be quite different for normal cells, which enjoy a stress-free environment and the presence of survival signals to counteract any activation of the death programme, compared to the labile state of cancer cells. The concept that apoptosis is the cumulative response to many signals, indicates that loss of any of these signals might enhance survival by reducing the apoptotic burden below the threshold that is necessary for the execution of death, thus enabling tumour cells to continue growing [48]. Conversely, this situation also means that adding an extra insult might tilt the response towards apoptosis in tumour cells, while remaining harmless in normal cells. Despite their toxicity, chemotherapy and irradiation obviously show some selectivity, which can be attributed to the apoptosis-primed state of tumour cells. Interestingly, molecular studies have demonstrated that apoptotic pathways are in part redundant [142-144], eliciting research to design therapies tailored to particular tumour types acting on still-active components.

The death-receptor (DR) mediated apoptosis programme provides a target for more tumour-specific therapy. Death factors such as FasL and TNF-related apoptosis-inducing ligand (TRAIL) are primarily involved in the apoptosis-inducing activities of immune effectors such as natural killer cells and cytotoxic lymphocytes against virus-infected or oncogenically transformed cells. Some of the death receptors, however, are not able to transduce signals. Instead, they seem to act as decoys that compete for the interaction of death factors with their signalling receptors [145]. In humans, two signalling death receptors, DR4 and DR5, and two decoy receptors, DcR1 and DcR2, have been identified. For unknown reasons, although the death receptors are expressed ubiquitously, normal cells are enriched for decoy receptor expression. As a result, soluble TRAIL induces apoptosis in a broad spectrum of human cancer cell lines both *in vitro* and *in vivo*, as well as in primary tumour cells derived from patients [146]. Like TRAIL, monoclonal antibodies that functionally engage DR4 or DR5 also show antitumour activity in certain xenograft studies. Most normal human cell types tested so far, including epithelial, endothelial, fibroblastic and smooth muscle cells, were not affected by TRAIL. Some other normal cell types, such as astrocytes, hepatocytes and keratinocytes, however, were sensitive to apoptosis induction by TRAIL under certain circumstances. Several recombinant versions of TRAIL have been generated in the hope for a less toxic variant and are currently under preclinical assessment [147].

Targeting telomerase

The fact that cancer cells are dependent on telomerase-mediated rescue of short telomeres has initiated the development of telomerase inhibitors to selectively target tumour growth. Anti-telomerase oligonucleotides have been shown to effectively inhibit proliferation of cancer cells *in vitro,* and similar oligomers have demonstrated low toxicity and reasonable bioavailability in humans [148]. However, since telomerase expression is not exclusively restricted to tumour cells, some side effects can be anticipated, although stem cells deep in the renewal tissues proliferate only intermittently [149] and typically have longer telomeres than tumour cells. Nevertheless, preclinical data should preclude detrimental effects to stem cells prior to clinical phase entry. Furthermore, telomerase inhibitors are unlikely to produce acute anti-proliferative effects due to the lag-time needed for telomere erosion. Rather telomerase inhibitors are expected to be most effective in combination with or as adjuvant to other conventional or experimental cancer treatments [150]. Alternatively, approaches have been designed to exploit the relative cancer-specificity of telomerase expression without targeting telomerase itself. These include the use of the hTERT promoter to selectively allow viral replication of a lytic virus or to drive the expression of toxic genes (see section "Cancer gene therapy") [150]. In addition, hTERT represents a nearly universal tumour-associated antigen that has been shown to elicit cytotoxic T lymphocyte responses in patients [151-153], and a vaccination protocol using autologous dendritic cells loaded with a hTERT-peptide is now tested in phase I clinical trials [154].

Anti-angiogenesis

Cancer cells begin the recruitment of new blood vessels early in tumoriogenesis [155]. The ensuing continuous presence of angiogenic factors prevents the tumour endothelium from maturing into a developed vessel network, and tumour vessels show an abnormal morphology, with an irregular diameter, thin walls and leakiness. The relative deficiency of supporting cells such as pericytes could also contribute to these morphological features in tumour vasculature. Importantly, tumour and normal vasculature differ also at the molecular level [156]. The tumour-specific angiostatic effects of compounds such as Endostatin and Angiostatin, first described by Folkman, are thought to act on these molecular differences [157]. A recent dose-finding trial with human Endostatin (rh-Endo) in patients with refractory solid tumours had variable effects on tumour blood flow and caused a significant increase in endothelial and tumour cell apoptosis, but clear anti-cancer effects were not observed [158].

Another potential target for anti-angiogenic therapy is VEGF, an important mediator of angiogenesis and maintenance of tumour vasculature. Recently, a specific antibody called bevacizumab (Avastin) has been developed to neutralize this mediator. Phase II clinical trials for metastatic breast and colorectal cancer showed moderate toxicity, including hypertension and thrombosis. Objective responses were uncommon (~ 9%), but significant prolongation of life after treatment was observed [159].

Yet other potential angiogenesis targets are the matrix metalloproteases (MMPs), proteins involved in multiple aspects of tumour physiology including tumour cell metastasis and endothelial cell invasion [127]. Unfortunately, there are more than 20 different MMPs and only limited data on their individual targets, distribution and contribution to carcinogenesis. Together with the absence of efficacy markers, these complications likely contributed to the disappointing results seen in human trials of MMP inhibitors (MPI) so far. Although a significant survival benefit was reported for patients treated with marimastat, another trial was prematurely terminated because the MPI seemed to stimulate disease progression in patients with pancreatic and small-cell lung cancer. Hopefully, newly gained insights in the mechanisms of MMPs will allow a better design of the current trials with MPIs [160], or lead to more specifically-tailored drugs.

Survivin

Survivin is a bifunctional protein that suppresses apoptosis and regulates cell division [161]. Its expression is among the most tumour-specific of all human gene products, rendering survivin a potential drug target [162]. Although survivin is expressed during normal foetal development, the expression is barely detected in terminally differentiated adult tissues except for testis, thymus and placenta. In contrast, it is abundantly expressed in a wide variety of malignant tissues. Although its precise function and mechanism of action remain to be elucidated, survivin was shown to be a member of the inhibitor of apoptosis protein (IAP) family, and its binding to and inactivation of active caspase-9 is dependent on phosphorylation of survivin on threonine 34 (T34) [163,164]. Interestingly, a mutant of survivin in which T34 is mutated to nonphosphorylatable alanine (T34A) disrupts cell division and induces apoptosis when overexpressed in tumour

cells [165], presumably by competing with endogenous survivin for access to the kinase, thereby preventing the phosphorylation of the wild-type survivin protein or other crucial substrates of the same kinase. Treatment of subcutaneous breast cancer xenografts with replication-deficient adenoviruses (see section "Cancer Gene Therapy") expressing survivin(T34A) resulted in an anti-tumour effect that surpassed that of the drug paclitaxel [165]. *In vitro*, normal fibroblasts, smooth muscle cells and endothelial cells proved refractory to survivin(T34A)-expressing adenovirus [165], presumably because endogenous survivin expression in normal cells is minimal. After VEGF stimulation, survivin is significantly upregulated in endothelial cells, suggesting that it has a function in normal cells as well. Possibly, tumour cells are more dependent on the anti-apoptotic stimulus of survivin than are healthy cells.

Recently, a survivin-derived peptide was found to be a tumour-specific antigen, capable of eliciting a specific CTL response *in vitro* [166]. In addition, it was shown that the promoter regulating survivin expression is selectively upregulated in tumour cells compared to normal cells, advocating its use in cancer gene therapy to drive tumour-specific expression of toxic genes or in the creation of a tumour-selective replicative oncolytic virus (see section "Cancer gene therapy") [167,168].

Melanoma differentiation-associated gene-7

Melanoma differentiation-associated gene-7 (*mda-7*) was identified with subtraction hybridization as a gene that is upregulated during terminal differentiation of human melanoma cells. Subsequently, it was shown that *mda-7* expression is lost during progression of melanoma, suggesting that downregulation of *mda-7* facilitates progression to invasive and metastatic changes [169]. Ectopic expression of *mda-7* by means of a replication-defective adenovirus induced apoptosis selectively in diverse human cancer cells *in vitro* and *in vivo* by p53-independent activation of caspases -9 and -3. In contrast, normal human fibroblasts or epithelial cells were unaffected by *mda-7* expression [170-172].

It has been suggested that MDA-7 exploits a hyperactive Ras/MAPK pathway to induce transcription of the growth-arrest and DNA-damage (GADD) family of genes. This requirement would explain why the apoptosis induction is specific for tumour cells and why, for instance, immortalized melanocytes are resistant to overexpression of *mda-7* [173]. However, another group showed that adenoviruses expressing mda-7 specifically induce and activate the double-stranded RNA-dependent protein kinase (PKR), a mediator in antiviral defence, which phosphorylates the α-subunit of eIF-2, resulting in termination of translation of viral transcripts [174]. PKR also appears to mediate anti-tumour activity through the activation of multiple transduction pathways involved in growth inhibition and apoptosis induction including NF-kB, p53, MAPK and FADD [175-177]. Chemical inhibition of PKR or deletion of the PKR gene by targeted disruption leads to inhibition of MDA-7-induced apoptosis, suggesting that the activation of PKR and its downstream targets may constitute a critical pathway for MDA-7-mediated apoptosis [174]. While the mechanism awaits further elucidation, the adenoviral vector expressing the *mda-7* gene is currently undergoing Phase I clinical trials because of its tumour-selective killing capacities. So

far, it has been found to be safe with only mild toxicities and phase II clinical trials have been initiated to determine its efficacy [178].

Heat Shock Proteins

Heat shock proteins (HSPs) and their co-chaperones have a critical role both in the recovery of cells from stress as well as in cytoprotection, guarding cells from subsequent insults. Chaperones recognize unstructured regions of proteins and exposed hydrophobic stretches of amino acids, and subsequently sequester or refold stress-denatured proteins, preventing their irreversible aggregation with other proteins in the cell. In addition to their role in protecting cells from stress, nearly all HSPs are constitutively expressed under normal growth conditions, where they function to maintain protein homeostasis by regulating the quality control of protein folding. Interestingly, cells that have lost their ability to regulate cell growth, such as tumour cells, often express high levels of multiple HSPs compared with their normal parental cells [179]. Depletion of Hsp90 by geldanamycin or of Hsp70 by antisense methods caused growth arrest or cell death in transformed cells, but not in their non-transformed counterparts [180-182]. Thus, high expression of these chaperone proteins appears to be a prerequisite for the survival of human cancer cells of various origins, although why this is beneficial has yet to be clearly established. One possibility is the ability of chaperones to suppress and buffer mutations that occur during the transformation process, a characteristic which could promote cell viability and even enhance the growth of otherwise mutant cells. In that sense, HSP upregulation may be a hallmark of cancer complementary to genome instability, because it allows cells to survive a high load of nonsense mutations, thereby increasing the chances for the occurrence of an advantageous mutation.

Because of their potential role in tumorigenesis, HSPs have been selected as targets for molecular therapy. For example, the Hsp90 inhibitor geldanamycin exerts its anti-tumour effect by inhibiting the intrinsic ATPase activity of Hsp90, resulting in degradation of Hsp90-client proteins via the ubiquitin proteasome pathway. 17-allylamino-17-demethoxygeldanamycin (17AAG), a geldanamycin derivative, showed good activity and cancer selectivity in preclinical models [183,184] and has now progressed to Phase I clinical trials [185]. Second generation Hsp90 inhibitors are being designed to overcome some of the drawbacks of 17AAG, including limited oral bioavailability and solubility. Compounds that target specific functions of Hsp90 may provide even greater clinical benefit, and may also contribute to understanding the tumour-selective nature of this therapy [186].

Cancer gene therapy

Viruses have been of interest to tumour biologists for decades for a variety of reasons. Initially, the existence of oncogenes was revealed by analysis of molecular interactions of proteins from tumourigenic viruses like the Rous sarcoma virus and simian virus 40. Since then, several viral genes have proven useful in the elucidation of normal and tumour cell signalling. In addition, viruses have been employed as transfer vehicles for the delivery of genes *in vivo*. Currently, the

field of cancer gene therapy encompasses a range of strategies and technologies, from direct killing of tumour cells based on the intracellular signalling pathways that have gone awry in these cells, to eliciting an immune response to tumour antigens. Immunotherapy of cancer depends on complex interactions between tumour cells and the immune system, and comprises a distinct intellectual challenge that is beyond the scope of this thesis. The former approach, eliminating tumours by direct transduction of genes or viruses, comprises three main strategies: the compensation of mutations in tumour suppressors and oncogenes, the exploitation of bystander effects in so-called molecular chemotherapy and tumour-selective replication. Notwithstanding recent advances in the delivery of genes by non-viral methods or other viral vectors such as the herpes simplex virus, I will restrict this paragraph to replication-deficient and –competent adenoviral vectors.

Replication-deficient vectors

The most frequent target for mutation compensation has been p53. Viral vectors can be used to introduce the healthy *p53* gene into cancer cells in order to induce cell cycle arrest and apoptosis. This approach has been widely tested in human trials, and although the safety data were excellent, in most cases tumour transduction has been insufficient to achieve clinical responses [187].

The classic example of molecular chemotherapy or gene-directed enzyme prodrug therapy is the transfer of Herpes Simplex virus type I thymidine kinase (TK), which catalyzes the phosphorylation of systemically administrable non-toxic ganciclovir (GCV) into a toxic metabolite that frustrates DNA replication [188]. Importantly, GCV-triphosphate can escape producer cells via gap junctions and by this means create a bystander effect, allowing cells not directly transduced by the vector to be killed [189]. In a clinical trial for glioma, patients were randomized to receive Ad-TK, packaging cells for retrovirus with TK or Ad-LacZ [190]. The patients injected with Ad-TK survived significantly longer than the other groups, suggesting that clinical responses can be achieved even with this relatively basic approach. Considering the highly variable expression of the receptor used for adenoviral internalisation (CAR) on human primary tumours, it seems likely that efficacy could be further increased with the enhancement of infectivity [191-193], perhaps rendering this approach feasible for the local treatment of small tumour nodules. An important consideration remains that TK-GCV is not tumour-specific, instead it targets dividing cells, as well as mitochondrial DNA, thereby eliciting toxicity in non-dividing but mitochondria-rich hepatocytes [194]. Thus for this approach targeting of the vector and/or expression of the gene would be advisable.

Conditionally-replicating viruses

An especially promising approach in cancer gene therapy is the use of replication-competent viruses. Their development has been based on the need for more effective tumour penetration. In theory, subsequent rounds of replication enable these viruses to penetrate deeper into the tumour until all of the cells have been infected. Each infected cell can produce thousands of

virions and therefore cause a substantial amplification of the infection, combined with a more effective antitumour effect if a lytic virus is used. Obviously, it is crucial to limit the replication of the agent to tumour cells. Hence, the identification of the 'cancer hallmark pathways' provided new possibilities for the development of conditionally-replicating adenoviruses (CRADs). Both adenovirus replication and tumour cell proliferation require the disabling of similar restraints, namely to relieve inhibitions on cell division and to evade apoptosis (figure 5). The fact that tumour cells have already inactivated some of the inhibitory factors for adenoviral replication allows for the design of tumour-selective adenovirus replication. For instance, both adenoviruses and tumour cells require a block in p53 function to replicate or proliferate efficiently. Adenoviruses eliminate p53 by producing E1B55K, a protein that binds to p53 and, together with another viral protein E4orf6, targets it for destruction [195]. An attenuated adenovirus called mutant dl1520 or ONYX-015 lacks E1B55K and therefore cannot destroy p53. In normal cells, p53 blocks adenoviral replication, but in cancer cells that lack p53, ONYX-015 can replicate. As adenoviruses are lytic, cells allowing replication die in the process.

The specificity of ONYX-015 for p53-deficient cells has been questioned. It was demonstrated that ONYX-015 replicates in some tumour cells that retain wild-type p53 [196]. However, as stated earlier, most tumour cells that retain wild-type p53 are likely to have other defects in the p53 pathway. For instance in tumour cells that lack ARF, p53 is not activated during ONYX-015 infection, and therefore cannot block replication. Re-introduction of ARF in such cells induced high levels of p53 and consequently inhibited ONYX-015 replication but not wild-type adenovirus replication [197]. Furthermore, ONYX-015 does not always replicate efficiently in tumour cells that lack p53. This inefficiency may be due to additional functions of E1B55K for the virus other than blocking p53. These functions affect selective export and translation of viral mRNAs during infection. Some tumour cells (such as C33a cervical carcinoma cells) complement these other functions of E1B55K better than others (such as U2OS osteosarcoma) [198]. Recently, adenovirus mutants have been generated that specifically target the p53 binding function of E1B55K while preserving its role(s) late in the viral life cycle [199]. In cell culture studies, these viruses demonstrated a replication capacity that surpassed that of ONYX-015.

While *in vitro* studies were carried out to obtain more information on the potency and selectivity of ONYX-15, clinical studies were already initiated. Treatment of patients with head and neck cancer revealed that the agent is safe, but objective tumour responses were seen in fewer than 15% of the patients. Most likely, the clinical response has been limited by poor distribution of the injected virus, which could be due to the high proportion of stromal and fibrotic tissue in these tumour types, and/or to insufficient complementation of the additional E1B55K functions. In contrast to its limited effects as a single agent, ONYX-015 has shown promising results in combination with chemotherapeutics. After treatment with ONYX-015 combined with 5-fluorouracil and cisplatin, objective responses (>50% reduction in tumour size) were seen in 63% of patients with recurrent head and neck cancer, and 26% had complete responses. A Phase III trial is now in progress [200].

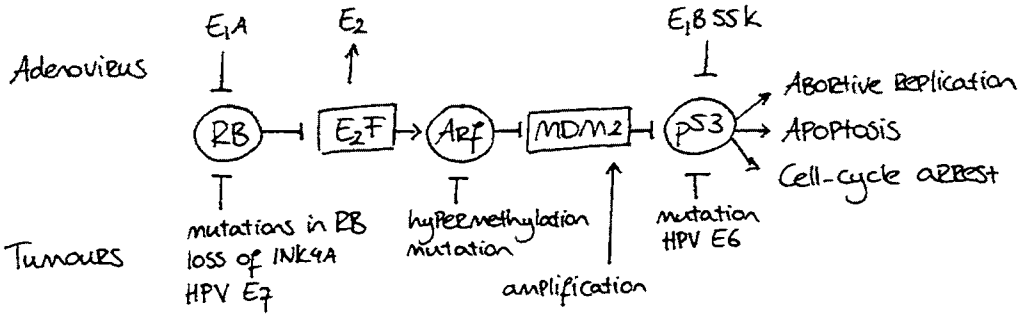

Figure 5. Common alterations in the RB and p53 pathway in adenovirus-infected cells and tumours.

Adenovirus E1A binds the tumour suppressor RB, thereby preventing it from repressing E2F. Subsequently, E2F activates host target genes that are involved in the S-phase of the cell cycle and the adenovirus E2 region. In tumours, the RB pathway can be inactivated in various ways, for instance by mutation of RB or inactivation of INK4A (see section "Growth control"). In addition, protein E7 of the human papillomavirus (HPV), implicated in cervical cancer, can bind and inhibit RB. One of the genes that E2F activates is Arf, an inhibitor of the oncoprotein MDM2 (see section "Cell suicide"). Inhibition of MDM2 results in upregulation of p53, which prevents both viral replication and tumour progression. Adenovirus E1B 55K binds and inhibits p53 so that replication can proceed. P53 itself is mutated or deleted in around 60% of tumours and can be targeted for destruction by HPV E6 protein. Additionally, inactivation of Arf or upregulation of MDM2, resulting in loss of p53 function, are also found in human cancer. Adapted from ref 198.

Another adenovirus protein, E1A, allows for the targeting of the RB pathway, as E1A binds and neutralizes RB and its relatives. As described, tumour cells need to inhibit RB to activate the transcription factor E2F, which in turn drives expression of genes involved in S-phase. Adenovirus, on the other hand, has hijacked E2F to activate expression of the E2 region of the viral genome. Thus, normal cells with functional RB should be resistant to E1A-defective viruses, whereas malignant cells should permit replication. Several E1A-defective viruses have been described and tested in preclinical models [201]. Early generation viruses, although not replicating in normal cells, still expressed some viral genes and thereby caused toxicity. Newer vectors not only contain a deletion within the E1A-CR2 region (essential for RB binding), but are more stringently dependent on RB inactivation due to replacement of the viral E1A and E4 promoter regions with the human E2F1 gene promoter.

The combination of these features in this virus called ONYX-411 was essential for the selective killing of tumour cells *in vitro*, reduced *in vivo* systemic toxicity, and conveyed a survival benefit to mice carrying human breast cancer xenografts following systemic administration [202]. Although E1A has additional functions, such as binding p300 and activating the E4 region of the genome, *in vitro* studies demonstrated that ONYX-411 had a higher cell killing capacity than did ONYX-015, suggesting that the additional E1B55K functions are more important for viral replication than are the additional E1A functions.

The potency of CRADs can be further increased by engineering them to overexpress the adenovirus death protein (ADP): this increases cell lysis and viral release, rendering the virus more active *in vivo* than an E1A mutant alone. Viruses that exploit these features are under development [198]. Moreover, studies in preclinical models demonstrated that the oncolytic effect of CRADs directly correlates with infectivity. Therefore, the replicative potential and tumour-specificity of CRADs are important determinants not only of their clinical efficacy, but also of their efficiency of progeny release and re-infection [200].

An alternative approach to CRADs is the control of adenoviral-essential genes by tissue- or tumour-specific promoters. For example, the promoter/enhancer elements for prostate-specific antigen, L-plastin and α-fetoprotein, have been used to achieve selective expression in prostate, breast and hepatocellular carcinomas respectively [203-205]. In contrast, control of E1A expression by the E2F promoter targets a wide range of tumour types. So far the efficacy of this tumour/tissue-specific promoter approach has been limited, as it is dependent on the promoter activity in target tumours and in normal tissues, which leads to reduced efficiency of viral replication in the tumour and reduced specificity [206].

Viruses + Cancer

Tumour-tropism of viruses

For one reason or another, many viruses have a natural tropism for tumour cells. It has been suggested that some RNA viruses take advantage of tumour-associated defects in the interferon response pathway. As an antiviral defence, cells activate the kinase PKR, resulting in termination of translation of viral transcripts. However, in cells with an activated Ras signalling pathway, as is the case for most tumour cells, PKR kinase activity is impaired, allowing viruses such as reovirus, vesicular stomatitis and probably also the Newcastle disease virus to replicate [206-208].

Parvoviruses were first thought to be carcinogenic because they were isolated from tumours. However, they turned out to have tumour-tropism due to the fact that in normal cells, the single-stranded viral genomes trigger a p53-dependent DNA-damage response that blocks cells in G2, preventing replication. Hence, replication in p53-deficient tumour cells will be more efficient [209]. In addition, free E2F, occurring in replicating cells and in cells with a deregulated RB pathway, stimulates parvovirus promoter activity [210]. Although the precise mutations targeted by parvoviruses are unknown, it was shown that malignant transformation increases the sensitivity of these cells to killing by the non-structural proteins of parvovirus H-1 [211,212]. By definition, the presence of these viruses in tumour masses demonstrates that they are not potent enough to kill all tumour cells [213]. But their tumour-selective replication renders them suitable as replication-competent vectors for more toxic genes.

Viruses as a source of anti-tumour activities

Adenovirus type 2 (Ad2) early region 4 ORF 4 (E4orf4) is a small 14-KD Ad protein that has multiple functions during Ad infection. In mammalian cells, expression of Ad2 E4orf4 triggers a p53-independent cell death programme that is restricted to transformed cells. Over 40 human cancer cell lines representing most major classes of human tumours were sensitive to E4orf4-induced cell death. In addition, E4orf4 had little effect on primary human cell types derived from various tissues. The effect of E4orf4 on tumour growth *in vivo* was studied by injection of a tetracycline-inducible E4orf4 adenovirus vector in human tumour cell lines xenografted on nude mice. In two different cancer cell types, expression of E4orf4 caused a reduction in tumour size more significant than that obtained using an adenovirus vector expressing p53 [214].

Cell death induced by Ad2 E4orf4 is associated with classical apoptotic hallmarks, including nuclear condensation, cell shrinkage and externalization of phosphatidylserines [215]. However, caspase activation in E4orf4-induced apoptosis seems to be required in some cell types but not in others [216], suggesting that E4orf4 may induce multiple death programmes.

Initial studies demonstrated that binding of E4orf4 to a B subunit of protein phosphatase PP2A, causing partial dephosphorylation of certain transcription factors, was important for the induction of cell death in tumour cells. Recently, in Saccharomyces cerevisiae, E4orf4 was shown to target PP2A to the anaphase-promoting complex/cyclosome (APC/C), leading to APC/C inactivation. As a result, yeast cells were arrested at the G2/M phase of the cell cycle [217]. Similarly, E4orf4 can induce G2/M arrest in mammalian cells prior to apoptosis. However, mutational analysis demonstrated that binding to PP2A is necessary but not sufficient for the induction of apoptosis in mammalian cells, indicating that E4orf4 possesses one or more additional functions required for cell killing. Gingras et al. found that Ad2 E4orf4 induced a Src-mediated cytoplasmic apoptotic signal requiring tyrosine phosphorylation of E4orf4, which rapidly led to caspase-independent membrane blebbing and cell death [218]. A separate paper demonstrated an additional, Src-independent nuclear cell death induced by E4orf4 independent of E4orf4 phosphorylation [219]. No clinical data on E4orf4 have been described yet, but further elucidation of the activity of this tumour-specific viral protein could contribute to our knowledge of tumour genesis.

Apoptin

The identification of tumour-specific pathways as described in the first part of this chapter provided an explanation for the existence of proteins with tumour-specific activities, which in turn led to the development tumour-specific therapies. That targeting of these oncogenic pathways can indeed lead to clinical success is demonstrated by the inhibition of tyrosine kinases. Approaches based on other pathways as exemplified by farnesyl transferase inhibitors, TRAIL and CRADs, indicate that the scope of cancer-specific therapies can be greatly extended, hopefully culminating in efficacy and selectivity for different tumour types. Even though tumours share common alterations that can be targeted by a tumour-specific agent, it is unlikely that one

therapy, 'the magic bullet', will be effective against all tumour types for at least two reasons. First, tumour-type specific differences in achieving the different traits (genetic road-maps) render therapies based on a specific mutation rather than the outcome of the whole pathway suitable for only a subset of tumours. Second, the therapeutics are critically dependent on their pharmacokinetics and distribution efficiency in certain tissues. Thus, the development of multiple strategies remains necessary for the aim of treating all cancers, but the fact that these strategies can now be based on specific tumour characteristics will hopefully result in a complete arsenal of effective anti-tumour agents with dramatically reduced toxicities.

The demonstration that tumour-specific treatment has a molecular basis and is in fact feasible has given a boost to research involving proteins with inherent tumour-selective killing abilities, which has in turn expanded our knowledge of the hallmarks of cancer and provided potential anticancer candidates. Examples have been given of depletion of tumour-essential proteins such as survivin or heat-shock proteins, or the introduction of apoptosis-inducing proteins such as cellular mda-7 and viral E4orf4. Another protein that induces tumour-selective apoptosis, called Apoptin (apoptosis-inducing protein), was described by Danen-van Oorschot et al. in 1997. The current data on the mechanism of action of Apoptin will be described below.

Origin of Apoptin

The chicken anaemia virus (CAV) belongs to the family of *Gyroviridae.* It has a single stranded genome of 2.3 kb that is transcribed into a single, polycistronic mRNA of 2.1 kb encoding three proteins, VP1, VP2 and Apoptin (VP3) (figure 6). The genes for these proteins are partially or completely overlapping but have distinct open reading frames, resulting in three different proteins without sequence similarity [220]. No homologues in any other viruses or living organisms have been detected thus far. VP1 is 51.6 kD and thought to be the only capsid protein [221]. VP2 (24.0 kD) has been implicated in virus assembly [222]. A recent report describes VP2 to be a dual-specific phosphatase, capable of removing phosphate groups from both tyrosine residues and serine/threonine residues *in vitro* [223]. If and how this function is important in CAV biology is not yet clear. The function of VP3 (13.6 kD) for the virus also remains to be clarified. Mutation of the last 11 amino acids blocks CAV replication *in vitro*, suggesting that VP3 is essential for CAV replication (Noteborn, unpublished results).

In young chickens, CAV mainly targets haemocytoblasts in the bone marrow and immature T cells in the thymus [224]. Not much is known about the replication cycle of CAV or how it initiates the destruction of its target cells. Apoptotic bodies, however, were detected in thymus cells from CAV-infected chickens with electronic microscopy and in cultures of CAV-infected lymphoblastoid cell lines. Agarose gel electrophoresis of DNA extracted from thymuses of CAV-infected chickens and from infected cell lines showed the characteristic pattern of apoptosis-specific DNA laddering. Early after infection, Apoptin expressed by CAV shows a finely speckled distribution in the nucleus, co-localizing with chromatin. At the same time, it can be detected in

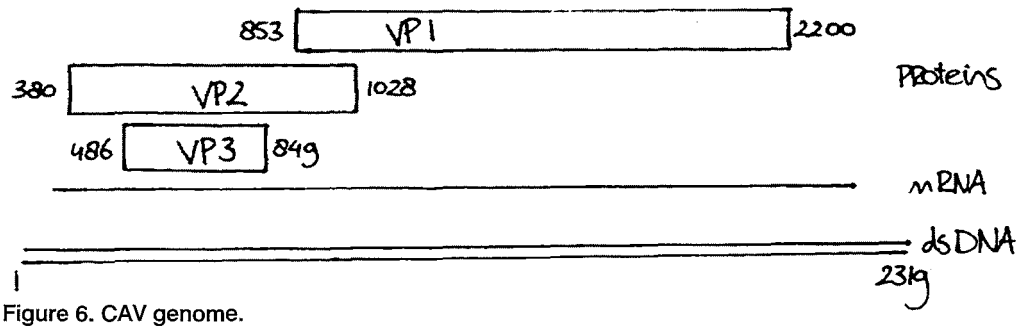

Figure 6. CAV genome.

The double-stranded, circular replicative intermediate of CAV is represented by the double lines. The single line depicts the polycistronic mRNA. The three open-reading frames VP1, VP2 and *Apoptin* (VP3) are indicated by square boxes, with the numbers designating the nucleotide positions.

perinuclear structures. During infection, Apoptin progressively concentrates into nuclear aggregates that partially co-localize with the apoptotic DNA [225]. In transfection assays *in vitro*, the expression of Apoptin alone was found to be sufficient for induction of apoptotic morphology and DNA laddering of lymphoblastoid cell lines [226]. Subsequently, Apoptin was shown to elicit apoptosis in a variety of human and rodent tumour cells as well [227].

Apoptin characteristics in tumour and normal cells

Apoptin is a protein of 121 amino acids that contains two nuclear localisation signals and a putative nuclear export signal (figure 7). The metazoan genome does not encode any proteins with homology to Apoptin. Because Apoptin is able to engage the apoptotic machinery in mammalian cells, probably via a functional interaction with cellular proteins, it is possible that analogues do exist. At present, these functional homologues are difficult to identify, as computer analysis of the Apoptin sequence predicts not much structure, a finding corroborated by circulair dichroism (CD) spectra [228]. Moreover, resolving the molecular structure of Apoptin by either nuclear magnetic resonance or crystallography has proved unsuccessful so far.

Currently, over 50 different tumour cell lines have been tested in transient transfection assays and all were sensitive to apoptosis induction by Apoptin. Remarkably, primary cells were not killed by Apoptin. These include human fibroblasts, keratinocytes, hepatocytes, mesenchymal- and $CD34^+$ haemotopoietic stem cells, endothelial and smooth muscle cells (ref 229, 230 and Zhang, unpublished data). Analysis has been restricted by the limited life span and by the inefficiency of transduction of these primary cells *in vitro*. However, expression of Apoptin in a limited number of cultured cells could be achieved by transfection or microinjection of DNA or recombinant protein and, in all cases, there was no, apparent apoptosis in the time span wherein all tumour cells had already perished due to Apoptin expression. Moreover, the localisation of Apoptin strikingly differed between tumour and normal cells; whereas Apoptin was expressed

1
MNALQEDTPPGPSTVFRPPTSSRPLETPHCREIRIGIAGITITLSLCGCANARAPTLRSAT
ADNSESTGFKNVPDLRTDQPKPPSKKRSCDPSEYRVSELKESLITT**T**PSRPRTAKRRIRL
121

Figure 7. Amino acid sequence of Apoptin.

Several slightly differing variants have been cloned worldwide; depicted is the one used in this thesis. Amino-acids 1-121 of Apoptin are shown in single-letter code, the putative NES is indicated by a single line, the putative NLSs are underlined by bold lines. The phosphorylation-target threonine 108 is enlarged and bold.

mainly in the nucleus of tumour cells it resided predominantly in the cytoplasm of normal cells. Redirecting Apoptin to the nucleus in normal cells, either by fusion to the SV40-NLS or direct microinjection of recombinant protein, did not result in apoptosis [231], Danen-van Oorschot, in preparation). In tumour cells, truncation of the C-terminal 11 amino acids of Apoptin, containing one nuclear localisation signal (NLS), strongly reduced the activity of Apoptin, and also changed its localisation from mainly nuclear to more cytoplasmic [229,232]. These data suggest that nuclear localisation of Apoptin is required for apoptosis induction, but is not sufficient.

Interestingly, microinjection of Apoptin into the nucleus of tumour cells resulted in delayed apoptosis, suggesting that Apoptin must go through the cytoplasm before entering the nucleus, possibly to target a cytoplasmic protein or acquire a necessary modification. In fact, it was recently demonstrated that Apoptin is post-translationally modified specifically in tumour cells. Metabolic ^{32}P-orthophosphate labelling revealed phosphorylation of Apoptin after ectopic expression in a variety of transformed and tumour cells, while no detectable phosphorylation of Apoptin by their normal cell counterparts was observed [233]. The phosphorylation was eventually determined to occur on threonine 108 (figure 7). A mutant Apoptin(T108E) was generated with a glutamic acid replacing threonine 108 to mimic constitutive phosphorylation and expressed in normal cells. The negatively charged amino acid at position 108 conferred both nuclear import and apoptosis induction in primary fibroblasts and mesenchymal stem cells. As expected, both wild-type Apoptin and a mutant Apoptin containing an alanine residue at position 108 (T108A) remained primarily in the cytoplasm and did not induce apoptosis in these cells [233]. These data indicate that the machinery needed for Apoptin to induce apoptosis is present in both tumour and normal cells, but that the kinase responsible for the phosphorylation of Apoptin is specifically active in tumour cells. However, when non-phosphorylatable mutants were expressed in tumour cells, cell death was reduced but still occurred (Rohn, unpublished observation), suggesting that a phosphorylation-independent pathway exists that enables Apoptin to induce apoptosis.

Indeed, mutational studies with Apoptin are also consistent with the idea that two separate death domains exist. Specifically, an Apoptin mutant consisting of the C-terminal amino acids 80-121, containing both NLS sequences and the phosphorylation site, localised to the nucleus and was capable of inducing apoptosis in tumour cells. In contrast, amino acids 1-69 of Apoptin displayed a cytoplasmic localisation when expressed in tumour cells and had mild apoptotic

activity. However, when fused to an SV40-derived nuclear localisation signal, the N-terminal domain of Apoptin induced robust apoptosis [231]and Danen-van Oorschot, in preparation). This domain could be responsible for the cell death observed with the non-phosphorylatable mutant of Apoptin. Indeed, when the N-terminus is deleted, the apoptosis induction by the C-terminal half of Apoptin is completely abrogated when the threonine residue is replaced by an alanine (Rohn, unpublished data).

The fact that normal cells are refractory to Apoptin, whereas Apoptin is capable of triggering apoptosis in all tumour cell types tested so far, suggests that a tumour-specific pathway allows the apoptosis induction by Apoptin. Which pathway this is remains to be established. It was shown that two to three days after transient expression of the SV40 large T antigen in primary fibroblasts or mesenchymal stem cells, Apoptin translocated to the nucleus and induced apoptosis, indicating that Apoptin responds to early changes in transformation and not to genetic alterations acquired later on. However, these experiments do not implicate which tumour hallmark pathway is responsible for Apoptin activation, as the activation could be a result of the inactivation of the RB pathway, the p53 pathway, or by any of the other functions of large T. The fact that CAV can induce apoptosis in normal chicken cells suggests that it could be a pathway employed under certain circumstances in normal cells as well, although further experiments are needed to determine whether Apoptin itself is actually responsible for the apoptosis observed in the context of the whole virus and whether viral infection exposes proteins/pathways that are not normally accessible in healthy cells. Intriguingly, CAV infection is frequently found concomitantly with transforming viruses such as Marek's disease [234], which may suggest that normal chicken cells are rendered more susceptible to CAV upon transformation.

Apoptosis induction by Apoptin

To obtain further insights in the downstream effects of Apoptin once it is activated, components of the cellular apoptotic machinery were inspected. Downstream caspases, like caspase-3, are activated during Apoptin-mediated apoptosis, but upstream caspases (1 and 8) do not seem to play a major role [235] (figure 8). In addition, Apoptin induces a slower form of cell death with atypical morphological changes in MCF7 cells (Noteborn, unpublished results), which lack caspase-3, suggesting that caspase-3 helps to execute a fast apoptotic death, but is not obligatory for Apoptin to be able to induce death. Thus, Apoptin seems to feed into the apoptotic pathway at the level of the downstream caspases, but how they are activated is still unclear.

As discussed earlier, most if not all tumours have a defective p53 pathway. However, some still possess wild-type p53 but have acquired mutations up- or downstream, allowing the activation of p53 by other mechanisms. Apoptosis induction by Apoptin, however, was not dependent on actions exerted by p53 because Apoptin also induces efficient apoptosis in tumour cells containing mutant p53 [236]. Additional studies indicated that Apoptin was not inhibited by overexpression of the anti-apoptotic proteins Bcl-2 and BAG-1 [237,238]. Remarkably,

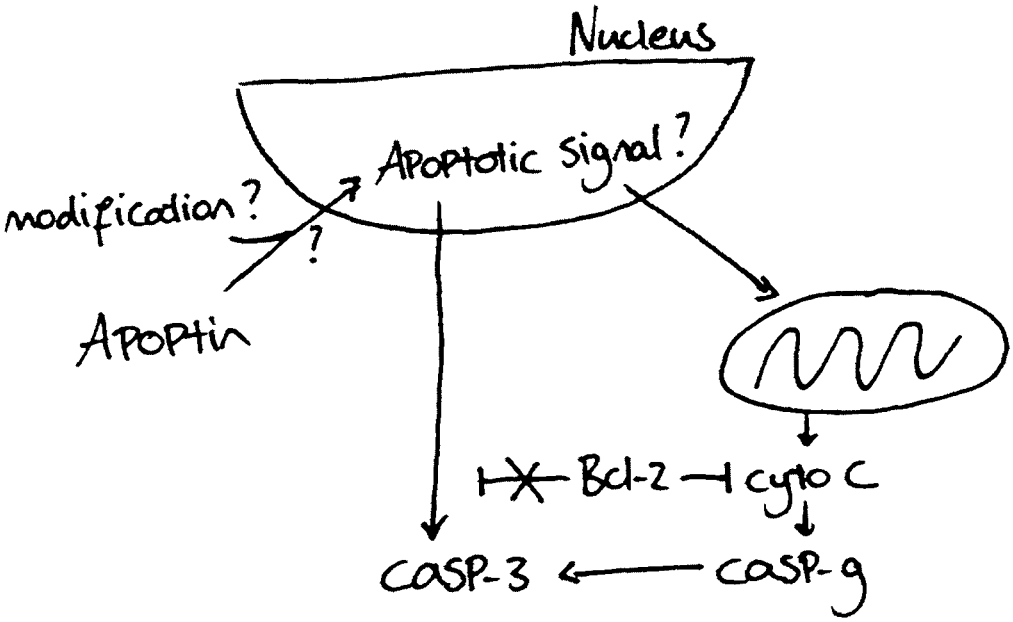

Figure 8. Model for induction of apoptosis by Apoptin.

Nuclear localisation of Apoptin seems to be required for its induction of apoptosis in tumour cells, but although its tumour-specific phosphorylation also conveys nuclear access, it is not certain if the essential apoptotic trigger is exerted in the cytoplasm or nucleus. Caspase activation and cytochrome c release are observed in Apoptin-induced apoptosis, albeit at a late stage, and as there is no inhibition by Bcl-2, Apoptin may also feed into the apoptotic pathway downstream of the mitochondria or activate a mitochondria-independent apoptosis pathway. Adapted from ref 231.

Bcl-2 even enhanced Apoptin-induced apoptosis at later time points [239]. During apoptosis, Bcl-2 can be cleaved into a pro-apoptotic fragment, which provides a positive feed-back loop but as a non-cleavable form of Bcl-2 was still capable of stimulating apoptosis by Apoptin, another mechanism must be involved [231].

Release of cytochrome c into the cytoplasm and loss of the mitochondrial membrane potential are observed only at a late stage in Apoptin-induced apoptosis, suggesting that these changes are the result, rather than the cause of cell death [235]. Together with the fact that Bcl-2 is unable to inhibit the Apoptin-induced apoptosis, this result suggests that initiation of cell death by Apoptin does not require the mitochondrial pathway.

Electron microscopy revealed that early after transfection, Apoptin was localised predominantly in the endoplasmatic reticulum (ER) of tumour cells (Mommaas and Danen-van Oorschot, unpublished results). At later time points it also becomes abundant in the nucleus. Release of Ca2+ from the ER can result in apoptosis [40,240,241], but it is unclear if this mechanism is involved in Apoptin-induced cell death. In fact, the ER-localisation of Apoptin could be due

merely to accumulation of misfolded Apoptin protein that must be properly folded by chaperones before it can enter the nucleus. Apoptin is highly insoluble, due to aggregation and 'stickiness'. Only fusion of Apoptin to a hydrophilic protein twice its size (maltose binding protein, MBP) gave rise to a generally soluble recombinant protein, whereas attachment of a His-tag conferred solubility only in specific buffer conditions. Microinjection of MBP-Apoptin protein in tumour and normal cells shows a localisation comparable to that of transfected Apoptin and the induction of tumour-specific apoptosis [230]. The recombinant MBP-Apoptin is organised in a stable multimeric complex consisting of 30 to 40 monomeric subunits as determined by scanning force microscopy and dynamic light scattering [228]. These complexes were found to specifically bind DNA in a cooperative fashion [242]. Interestingly, in the nuclei of intact cells Apoptin co-localises with both heterochromatin and nucleoli. In tumour cells with apoptotic morphology Apoptin co-localises with condensed DNA and ring-like structures in the nucleus, similar to the observations in CAV-infected transformed T cells. When MBP-Apoptin was co-injected with inhibitors of transcription and/or translation, apoptosis was still induced, indicating that Apoptin does not require *de novo* gene expression for its activity (Zhang, unpublished data). However, this does not rule out that repression of transcription is involved in Apoptin-induced apoptosis.

Cellular proteins associating with Apoptin

Apoptin also demonstrated different biochemical properties in tumour cells and their normal counterparts; the N-terminal epitope of Apoptin is readily accessible in tumour cells, whereas stringent lysis is required to detect it in normal cells [230]. Possibly, the N-terminus of Apoptin is bound to a cellular protein specifically in normal cells, or a different conformation of Apoptin shields this epitope in normal cells (see also chapter 6).

To obtain further insight in the mechanism of Apoptin-induced apoptosis a yeast two-hybrid screen was performed to identify cellular proteins that interact directly with Apoptin. For this, a cDNA library derived from Epstein-Barr virus-transformed human lymphocytes was used. After validating clones that were identified in the yeast screen by immunoprecipitations from human tumour cells, four known and six unknown proteins were found to be bonafide Apoptin-interacting proteins (Danen-van Oorschot, in preparation). One of the known proteins was DEDAF (death effector domain-associating factor) [243], a human protein found to associate with death effector domain (DED)-containing pro-apoptotic proteins. Its murine homologue RYBP has been shown to be a negative regulator of DNA transcription. DEDAF is expressed at low levels in a broad range of adult and fetal tissues, and has a strikingly high expression in placenta. Like RYBP, DEDAF was shown to bind the transcription factor YY1 and to repress transcription in a reporter assay. In overexpression studies, DEDAF localisation is predominantly nuclear, in both tumour and normal cells and its localisation is not altered when Apoptin is coexpressed. So in normal cells, where Apoptin resides in the cytoplasm, there is no co-localisation. However, in intact and apoptotic tumour cells partial co-localisation is observed, with distinct exclusion of DEDAF from nucleoli in contrast to Apoptin. Transient overexpression of DEDAF resulted in

apoptosis induction in various human tumour cell lines but not in primary cells. The tumour-specific apoptosis induction of DEDAF was very similar to that of Apoptin, in that it was also not blocked by Bcl-2 or CrmA, but is inhibited by p35 Danen-van Oorschot). The importance of DEDAF and other Apoptin associating proteins for the tumour selectivity and induction of apoptosis by Apoptin is currently being evaluated.

Scope of this thesis

Since cancer remains an unmet medical need, the development of novel anticancer therapies continues to be necessary. There is a need for agents potent enough to eliminate chemo- or radiation resistant cancer cells and this potency is intimately linked with their dose-limiting toxicity, i.e. tumour-cell specificity. As highlighted in this chapter, molecular therapy of cancer has proven to be a promising strategy. The study presented in this thesis investigates the potential applicability of Apoptin as an anti-cancer agent. To address this, we focussed on two approaches; gene therapy and transgenic mice. After generating a replication-deficient adenovirus as a more efficient method to deliver Apoptin, we were able to investigate for the first time the effects of Apoptin in tumours *in vivo*. In chapter 2, the generation of the gene-transfer vector is described. We demonstrate that this approach is technically feasible and that Apoptin remains tumour-selective when expressed by this vector, both *in vitro* and *in vivo*. Next, we examined short and long term effects of the Apoptin-adenovirus in human tumours xenografted on nude mice and show that it confers a significant survival benefit to the treated mice (chapter 3). Conventional cancer therapies fail to cure carcinomas of the bile duct, so in chapter 4 we analyzed whether Apoptin is potent enough to eradicate these therapy-resistant cancer cells. To obtain further insight in the potential toxicity of Apoptin in normal cells, especially after prolonged exposure and under different conditions, we generated Apoptin-transgenic mice (chapter 5). After characterisation of the expression patterns of Apoptin in these mice, we determined the effects of Apoptin in the cells with the highest expression, namely the lymphocytes. In addition, the biochemical properties of Apoptin in these mice hint at a possible (additional) mechanism for its tumour selectivity. Altogether, this thesis provides the first data on the effects of Apoptin *in vivo* and identifies directions for further development of Apoptin in tumour-specific therapy.

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Chapter 2

Specific tumor-cell killing with adenovirus vectors containing the Apoptin gene

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Abstract

Specificity is an essential prerequisite for cancer gene therapy. Recently we described that Apoptin, a protein of 121 amino acids which is derived from the Chicken Anemia Virus, induces programmed cell death or apoptosis in transformed and malignant cells, but not in normal, diploid cells (Danen-van Oorschot AAAM *et al., Proc Natl Acad Sci USA* 1997; 94: 5843-5847). This protein has an intrinsic specificity that allows it to selectively kill tumor cells, irrespective of the p53 or Bcl-2 status of these cells. Hence, it is attractive to explore the use of the Apoptin gene for therapeutic applications, *viz.* cancer gene therapy.

In this paper, we describe the generation and characterization of an adenovirus vector, AdMLPvp3, for the expression of Apoptin. Despite the fact that Apoptin ultimately induces apoptosis in the helper cells, which are transformed by the Adenovirus type 5 early region 1 (E1), the propagation kinetics and yields of AdMLPvp3 are similar to those of control vectors. Infection with AdMLPvp3 of normal rat hepatocytes in cell culture did not increase the frequency of apoptosis. In contrast, in the hepatoma cell lines HepG2 and Hep3b, infection with AdMLPvp3, but not with control vectors, led to a rapid induction of programmed cell death.

Experiments in rats demonstrated that AdMLPvp3 could be safely administered by intraperitoneal, subcutaneous or intravenous injection. Repeated intravenous doses of AdMLPvp3 were also well tolerated, indicating that the Apoptin-expressing virus can be administered without severe adverse effects. In a preliminary experiment, a single intratumoral injection of AdMLPvp3 into a xenogeneic tumor (HepG2 cells in Balb/C$^{nu/nu}$ mice) resulted in a significant reduction of tumor growth.

Taken together, our data demonstrate that adenovirus vectors for the expression of the Apoptin gene may constitute a powerful tool for the treatment of solid tumors.

Introduction

The applicability of cancer therapies is not only determined by their efficiency in eliminating tumor cells. Their specificity is equally important. Tumor-cell specificity allows the dose of the anti-cancer agent to be increased, thereby enhancing efficacy. Recently, *in-vitro* studies with the chicken anemia virus-derived protein Apoptin, demonstrated that this 121-amino-acid protein has an intrinsic tumor-cell specificity. It induces programmed cell death in immortalized and transformed cells, but not in normal, diploid cells. *In-vitro* studies have demonstrated that Apoptin induces apoptosis in a large panel of human transformed and malignant cells[1-3]. Many of the malignant cells exhibit alterations in their expression of proto-oncogenes and tumor-suppressor genes, e.g. *Bcl-2*[4] or *p53*[5], a phenomenon often associated with a poor response to chemotherapeutic agents[4, 6-8]. Remarkably, Apoptin-induced apoptosis is not dependent on functional p53[9], is not inhibited by *Bcr-Abl* expression and is even stimulated by overexpression of the apoptosis inhibitor *Bcl-2*[10]. An attractive feature of Apoptin is that it does not induce apoptosis in normal cells[3]. This is correlated with the subcellular localization of the protein. In normal cells, Apoptin is in the cytoplasm whereas, in tumor cells, it is found in the nucleus[3]. This suggests that Apoptin may be used as an agent that selectively eliminates malignant cells, provided that it can be delivered to target cells *in vivo* in sufficient amounts. One obvious strategy to reach this goal would be the application of the Apoptin gene, rather than the protein itself.

At present the most efficient system to achieve this makes use of adenoviral vectors. These vectors have several advantages that make them particularly suitable for *in-vivo* gene transfer. Recombinant adenoviral vectors (rAdVs) can be grown to high titers, have the capacity to transduce non-mitotic cells, and do not integrate their genomes into host-cell DNA. Moreover, adenovirus vectors have already been applied for clinical gene-therapy trials.

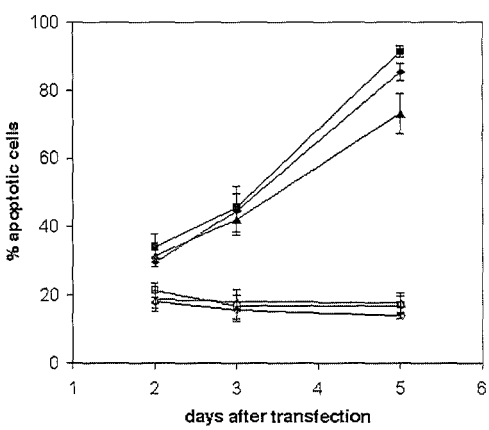

Figure 1. Apoptosis induction by Apoptin in adenovirus helper cell lines.

Cultures of 293 (square), 911 (diamant) and PER.C6 (triangle) cells were transfected with pCMV-vp3 expressing Apoptin (black) or with pCMV-desmin (open) as a control plasmid. The cells were fixed at several timepoints after transfection and analysed by indirect immunofluorescence. The percentage of cells that stained abnormally with DAPI was used as a relative indicator of apoptosis. Results are shown as the mean of at least three independent experiments, the error bars indicate the standard deviation. In each experiment at least 100 cells expressing Apoptin or desmin were examined.

In this paper, we describe the generation and characterization of an Apoptin-expressing adenoviral vector. We demonstrate that Apoptin maintains its specificity for tumor cells when introduced and expressed by an adenoviral vector. The intrinsic specificity and the inherent low toxicity make Apoptin adenovirus vectors promising tools for the treatment of solid tumors.

Results

The effect of Apoptin on helper cell lines

Most adenovirus vectors are E1-deleted and are therefore replication-defective. It is necessary to propagate them in Ad5E1-transformed helper cell lines, such as 293[11], 911[12] and PER.C6[13]. This entails a potential problem, since Apoptin induces apoptosis in transformed cells. One can, therefore, envisage that the helper cell line undergoes apoptosis due to the action of Apoptin, and that this may interfere with the formation of progeny vector particles. For this reason, the sensitivity of three helper cell lines to Apoptin-induced apoptosis was determined. Cell lines 293, 911 and PER.C6 were transfected with pCMV-vp3, an expression plasmid encoding the Apoptin gene[1]. At various time-points after transfection the cells were fixed, and the Apoptin protein was visualized by indirect immunofluorescence. To identify apoptotic cells, the cells were also stained with DAPI, which stains normal nuclei strongly, but apoptotic nuclei weakly and/or irregularly[14].

Apoptin induces apoptosis with equal kinetics in the three cell lines. The percentage of apoptotic cells reaches up to 80% after 5 days. However, 48 hours after transfection, about 25% of the cells expressing *Apoptin* were apoptotic (figure 1). This is just above the background level of apoptosis observed after transfection of pCMV-desmin, which encodes desmin as a non-apoptosis-inducing control[3]. In the following days, the frequency of apoptosis increased, just as observed for other transformed cells tested in transient transfection assays[2,3]. Since the lytic cycle of rAdVs is approximately 2 days, it is conceivable that an adenoviral vector expressing Apoptin can be generated in all of these helper cell lines.

Generation of a recombinant adenoviral vector expressing Apoptin

Recombinant adenoviral vectors are generated by co-transfection of an adaptor plasmid containing the gene of interest with a plasmid containing a cloned copy of a partial adenovirus (Ad) type 5 genome into helper cells. Upon homologous recombination between sequences present in the adaptor and in the adenovirus plasmids, an Ad genome is generated in which the Ad *E1*-region is replaced by the gene of interest. This *E1*-deleted vector can replicate in the helper cells by virtue of the E1 proteins provided *in trans* by the helper cells. The gene encoding Apoptin (vp3) was cloned in the Ad adaptor vector pMad5[15]. The resulting construct (pMad-vp3) is depicted in figure 2A. Similarly, Apoptin was inserted in the reverse orientation resulting in pMad-as, in order to generate a control virus that can be used in animal experiments to distinguish between the effects of the Ad vector and those of Apoptin. Since the minus strand of

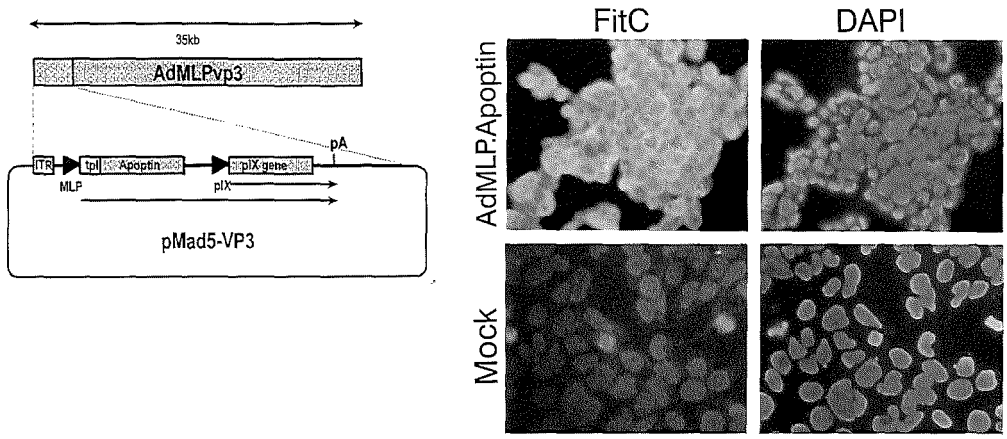

Figure 2. Characterisation of the adenoviral vector expressing Apoptin (AdMLPvp3).

(A) Schematic representation of the adenovirus vector. The backbone adenoviral sequences are derived from an *E3*-deleted adenovirus serotype 5, with a bacterial insert in the *E1* region. Following recombination, the *E1* region is replaced by the Apoptin coding sequences under the regulation of the Major Late Promoter (MLP) either in the sense orientation, e.g. AdMLPvp3 encoding Apoptin, or in the antisense orientation, e.g. the control virus AdMLPas. ITR: inversed terminal repeat, tpl: tripartite leader, pIX: structural protein of the adenovirus, pA: polyadenylation signal.

(B) PER.C6 cells producing AdMLPvp3 (upper panel) or mock infected (lower panel) were stained for Apoptin expression 24 hours after infection. Apoptin was stained with anti-Apoptin mAb 85.1 (Fitc) and the DNA was stained with DAPI (DAPI).

the chicken anemia virus is noncoding[16], no proteins will be expressed from the transgene of this control vector.

The recombinant adenoviral vector expressing Apoptin (AdMLPvp3) and the control viral vector (AdMLPas) were generated by cotransfecting 911 helper cells with the linearized Ad adaptor constructs (pMad-vp3 and pMad-as) and pJM17[17], containing an Ad5 genome lacking the *E1* and *E3* regions. The resulting plaques were isolated and after three rounds of plaque purification transferred to PER.C6 cells. These cells contain the E1 region regulated by a heterologous promoter which, combined with matched adaptor plasmids, eliminates the generation of replication-competent adenoviruses (RCA) by homologous recombination[12]. All batches of AdMLPvp3 were tested for Apoptin expression by indirect immunofluorescence (figure 2B) and for the presence of RCA by a PCR assay, using primers directed to the *E1* region giving rise to a band of 615 bp when RCA occurs (as described in the Materials and Method section). As expected, the AdMLPas control vector originating from pMad-as did not express Apoptin. All the virus batches used passed an RCA test detecting 1 pfu of an E1-containing adenovirus amidst 10^7 pfu of an *E1*-deleted vector.

To investigate whether the expression of Apoptin influences the virus yields, PER.C6 cells were infected with either AdMLPvp3 or AdCMVlacZ. The cells were harvested with 8-hour intervals. The growth curves of AdMLPvp3 and AdCMVlacZ are similar, indicating that Apoptin

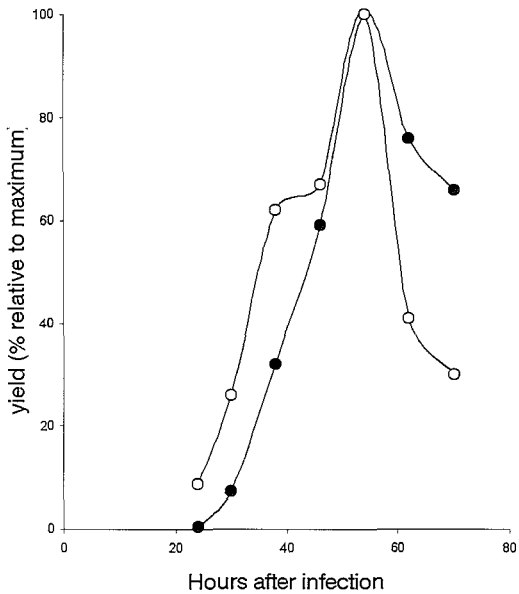

Figure 2(C) Yield of the recombinant adenoviral vectors.

PER.C6 cells were infected with AdMLPvp3 (moi 1.5, open circles) or AdCMVlacZ (moi 0.6, black circles). The cells and medium were collected at 8-hour intervals after infection and after 3 times freeze/thawing the virus titer was determined by plaque assay. The results are shown as the percentage of virus (pfu) in the cell pellet at a given time point relative to the highest amount of virus (pfu) found during the experiment

expression does not influence the vector yield (figure 2C). In all subsequent experiments, a harvest time of 48 hours was used.

Characterization of AdMLPvp3

To determine whether Apoptin maintains its tumor cell specificity also after adenovirus-mediated gene transfer, normal and transformed cells were infected with AdMLPvp3 and AdCMVlacZ. In view of the hepatotropic nature of human adenovirus type 5 after systemic delivery[18,19], we were particularly interested in the effect of Apoptin on liver cells, and liver-derived tumor cells. Therefore, HepG2 and Hep3b human hepatoma cells (with and without functional p53, respectively) were infected with AdMLPvp3 and after 24 hours expression of Apoptin was determined (figure 3A). After 24 hours, already 70-80% of the hepatoma cells expressing Apoptin are undergoing cell death (figure 3B). The background level of cell death is determined in parallel infections of the cells with AdCMVlacZ. The ability of AdMLPvp3 to induce cell death in these transformed cells is equal to that of pCMVvp3 (not shown).

So far, all normal cell types have been found to resist Apoptin-induced apoptosis. The cell types that have been tested by transfection of an expression plasmid include normal keratinocytes, fibroblasts, endothelial cells, smooth muscle cells and T cells[3]. To study what the effect of the Apoptin gene would be in normal cells when introduced by an adenoviral vector, primary rat hepatocytes were infected (figure 3A). After two days no difference was observed in the percentage of dead cells whether they expressed *Apoptin*, or *lacZ*, or after mock infection (figure 3B). These observations indicate that, in hepatocytes, the adenoviral vector does not influence the specificity of Apoptin.

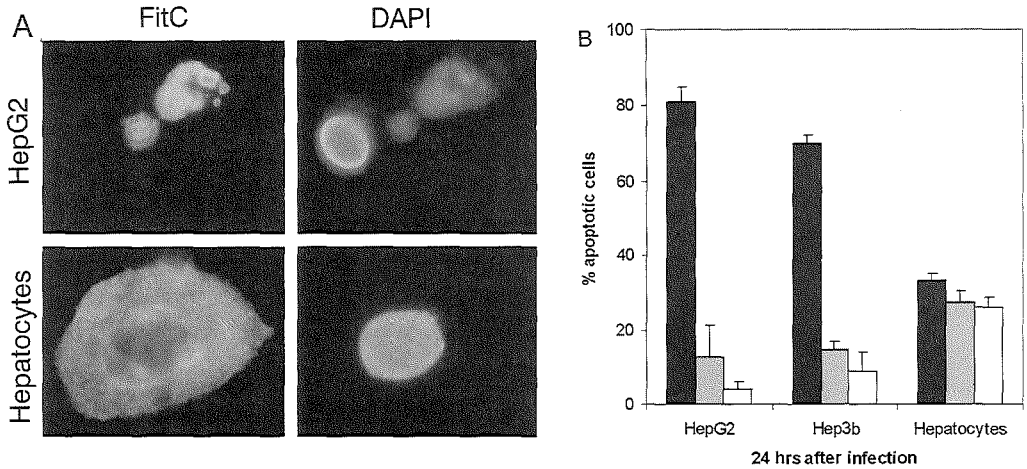

Figure 3. The effect of Apoptin on primary and malignant hepatocytes.

Primary rat hepatocytes and human hepatoma (HepG2 and Hep3b) cells were infected with AdMLPvp3 or AdCMVlacZ. For the hepatocytes a multiplicity of infection (moi) of 5 was used, the hepatoma cells were infected with moi 20 . (A) Forty-eight hours after infection the cells were fixed and analysed by indirect immunofluorescence (panel A). Apoptin was stained with mAb 85.1 (FitC) and the DNA was stained with DAPI (DAPI). (B) HepG2, Hep3b and primary rat hepatocyte cells were fixed 24 hours after infection. The percentage of cells that stained abnormally with DAPI was used as a relative indicator of cell death. The percentages are given for cells expressing Apoptin (black bars), β-galactosidase (grey bars) and non-infected cells. In each experiment at least 100 cells expressing Apoptin or β-galactosidase were examined.

The data also show that Apoptin maintains its characteristic localization after adenoviral transduction: nuclear in transformed cells and cytoplasmic in normal diploid cells (figure 3A). Moreover, transformed cells remain sensitive to Apoptin-induced apoptosis, whereas normal cells are not.

To further characterize the nature of AdMLPvp3-induced cell death we visualized the presence of DNA strand breaks with the aid of the enzyme terminal deoxynucleotidyl transferase and Fitc-labeled dUTP (TUNEL assay). HepG2 cells were infected either with AdMLPvp3 or with AdCMVlacZ and after 20 hours stained for the transgene to confirm similarity in transduction efficiencies (figure 4). After 40 hours, parallel-infected dishes were subjected to the TUNEL assay. Even though approximately 15% of the HepG2 cells were expressing β-galactosidase, only occasionally a single cell exhibited DNA breaks that could be detected by the TUNEL assay. However, the frequency of TUNEL-positive cells 40 hours after AdMLPvp3 infection appeared to be in the same range as the frequency of Apoptin positive cells 20 hours after infection.

The anti-tumor cell activity of AdMLPvp3 is also demonstrated in-vitro by the clearance of a dish HepG2 cells 48 hours after infection at a moi of 50. Giemsa staining visualises the number of remaining cells attached to the dish, compared to the number of cells after infection with AdCMVlacZ (figure 5). Inspite of a slight toxic effect of the adenoviral vector itself, as observed in the difference in cell density between the mock and AdCMVlacZ infected cells, these data

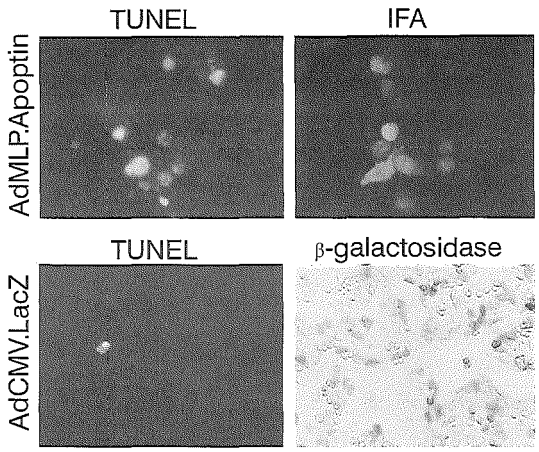

Figure 4. Apoptosis induction by AdMLPvp3.

Parallel dishes of HepG2 were infected with AdMLPvp3 and AdCMVlacZ. After 20 hours immunofluorescence was performed to detect the number of cells expressing Apoptin, and β-galactosidase assay was performed on the AdCMVlacZ infected cells to confirm comparable transduction efficiencies (10-15%). After 40 hours a TUNEL assay was performed on parallel dishes, using Fitc-labeled nucleotides.

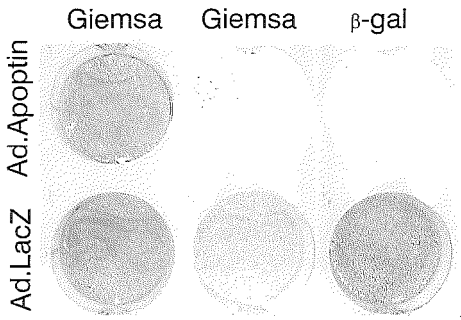

Figure 5. Effect of AdMLPvp3 on viability of HepG2 cells.

HepG2 cells were mock-infected (left dishes) or infected with AdMLPvp3 and AdCMVlacZ (moi 50). After 48 hours, one dish was stained with Giemsa and the other dish was used for a β-galactosidase assay.

confirm the potency of Apoptin in killing tumor cells. Thus, *in vitro*, the HepG2 cells are killed via apoptosis within 48 hours after infection with AdMLPvp3.

Anti-tumor effect of AdMLPvp3 on human hepatoma cells *in vivo*

To investigate the anti-tumor effect of Apoptin *in vivo*, 18 nude mice were injected with $1*10^7$ HepG2 cells subcutaneously in both flanks. After 22 days, when the mean tumor dimensions were 50 mm^2 (n=35), a single dose (equaling $8*10^9$ pfu in a total volume of 40 μl) of either AdMLPvp3 (n=12), AdMLPas (n=12), AdCMVLacZ (n=3) or saline (n=8) was injected intratumorally. In the following days, the tumor sizes were determined up to the nearest 0.5 mm with microcalipers and all animals were sacrificed at day 7.

To obtain an impression of the efficiency of virus distribution and tumor cell transduction after an intratumoral injection with an adenoviral vector, we performed a β-galactosidase staining on tissue sections obtained from AdCMVLacZ injected HepG2 tumors (figure 6). Since the tumors were isolated at the same time point (7 days after injection), the original percentage of infected cells must have been even higher. Due to the lobular growth of HepG2 tumors, it is difficult to obtain an even distribution of the virus throughout the tumor, which is also demonstrated by the lacZ staining patterns.

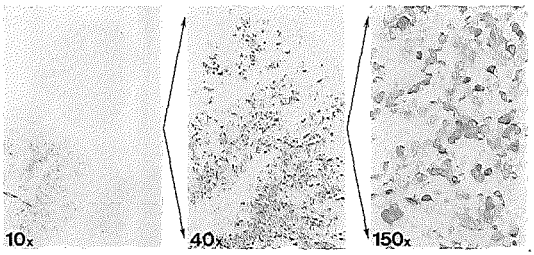

Figure 6. Transduction of HepG2 tumors after a single intratumoral injection with AdCMV-lacZ.

Paraffin sections of a HepG2 tumor 7 days after injection with $8*10^9$ pfu AdCMVlacZ, were stained with a polyclonal antibody directed to β-galactosidase.

Tumors treated with AdMLPvp3 showed markedly reduced growth, whereas tumors injected with the control virus or saline did not (figure 7A). Growth rate was significantly lower in the AdMLPvp3-treated group than that in the buffer-treated ($p<0.05$) and the AdMLPas-treated groups ($p<0.02$), as determined with a multiple comparisons test. The gene transfer into the tumors was confirmed by Southern blot analysis with the radioactively labeled Apoptin gene as a probe (figure 7C).

Visually, the HepG2 tumors injected with a single dose of AdMLPvp3 differed from the control tumors: the treated tumors had a pale appearance and seemed to contain vesicle-like structures (figure 7B). Histological examination of AdMLPvp3-treated tumors revealed relatively hypovascularised tumor tissue, when compared to tumors treated with AdMLPas or saline. It seems possible that the reduction of vascularization is due to an indirect effect, i.e. caused by a reduced production of factors stimulating angiogenesis due to the Apoptin induced cell death.

Toxicity of AdMLPvp3

To evaluate the possible toxic effects of AdMLPvp3, we injected $2*10^9$ pfu of virus (AdMLPvp3, AdCMVluc or saline) in healthy rats in three different manners: subcutaneously, intraperitoneally and intravenously.

Since body weight is generally a good indicator of health status within age-matched groups, we used this parameter as a criterion of well being. It can be seen in figure 8A that, after an initial drop in weight after injection, all groups gained weight similar to control animals (range within 10%). After one week, all animals were sacrificed.

To confirm efficient transduction of the cells of the liver parenchyma, DNA was isolated from the livers of all of three groups, and used for Southern analysis (figure 8B). These data revealed efficient transduction after i.v. administration (approximately 0.25 genome equivalent per cell was detected 7 days post infection), and, as expected, far less after i.p. and s.c. administration (less than 0.02 genome equivalents).

We previously observed an induction of a proliferative response of the liverparenchyma after systemic delivery of adenoviral vectors carrying different transgenes. We analyzed livers from animals intravenously injected with either saline, AdCMVluc or AdMLPvp3. BrdU labeling indices in liver parenchymal cells were similarly increased in both AdMLPvp3 and AdCMVluc treated rats compared to saline controls. This increase in BrdU incorporation was paralleled by a similar small increase in the frequency of apoptotic cells in both virus-treated groups (table 1).

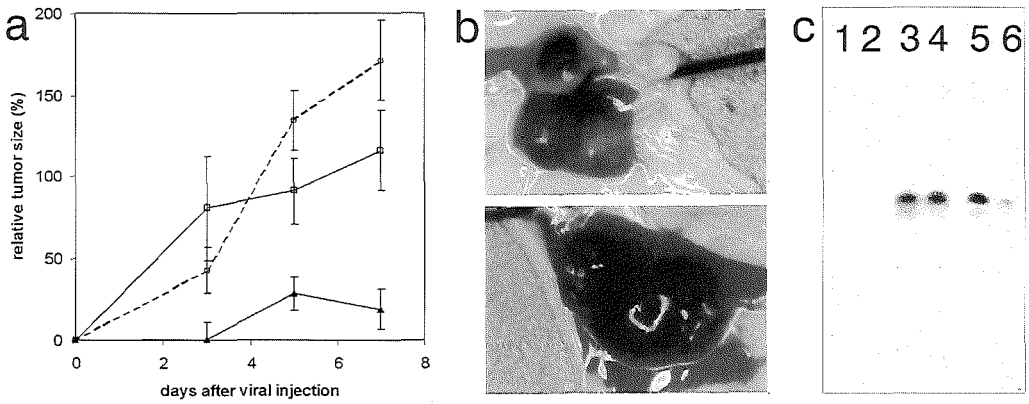

Figure 7. Anti-tumor effect of Apoptin.

Human hepatoma (HepG2) cells were subcutaneously injected at both flanks of 16 athymic nude mice ($1*10^7$cells/site). After 22 days the tumors were injected with a single dose ($8*10^9$ pfu) of AdMLPvp3 (n=12), AdMLPas (n=12) or saline (n=8). After 7 days the mice were sacrificed and the tumors harvested. **(a)** The rate of tumor growth is depicted relative to the initial size of tumors treated with AdMLPvp3 (black triangles), AdMLPas (open squares) or saline (open circles). The error bars indicate the standard error of the mean. **(b)** Treated tumors with the Apoptin expressing adenovirus vector (upper panel) harboured a less pronounced vascular bed compared to tumors treated with AdMLPas (lower panel) or saline (not shown). **(c)** Confirmation of gene transfer by Southern blot analysis with ^{32}P-labeled Apoptin gene. Lanes 1 and 2 contain DNA isolated from saline treated tumors, and lanes 3 and 4 DNA from tumors treated with AdMLPvp3. Lanes 5 and 6 contain DNA isolated from tumors treated with AdMLPas. In the AdMLPas the Apoptin gene is present in the reverse orientation. As a result also the DNA isolated from the tumors treated with this control virus yield a hybridizing signal.

Table 1

Kinetics of liver parenchyma cells were analysed for 6 rats, 8 days after iv injection with $2*10^9$ pfu of viral vector or PBS.

Treatment	Apoptotic cells	Mitotic cells	BrdU positive cells
PBS	0	0	18
PBS	0	1	22
AdCMV-luc	65	7	267
AdCMV-luc	6	2	78
AdMLP-vp3	90	3	195
AdMLP-vp3	37	0	263

The data are shown for the individual animals. Numbers represent the total number of BrdU-positive, apoptotic or mitotic cells in 100 randomly distributed areas, containing approximately 100 liver parenchymal cells each.

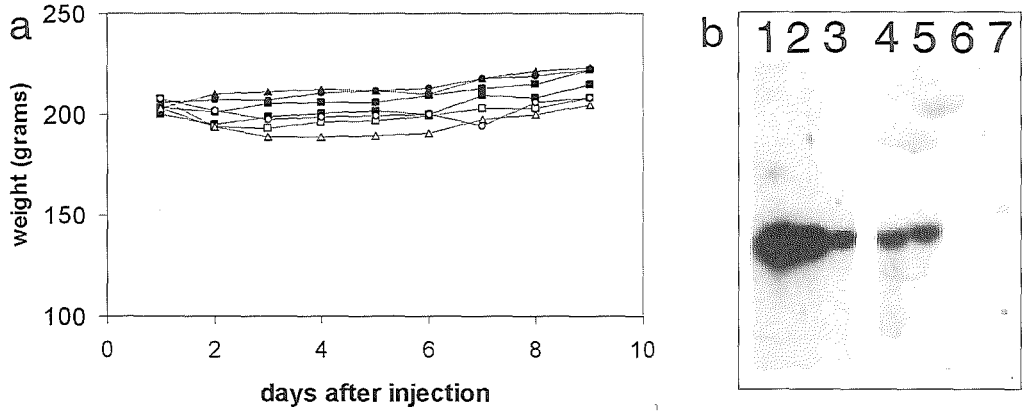

Figure 8. Toxicity of AdMLPvp3 in rats.

Healthy rats were injected with $2*10^9$ pfu AdMLPvp3 (n=6, open symbols) or AdCMVluc (n=6, black symbols) via three administration routes; subcutaneously (circles), intraperitoneally (triangles) or intraveneously (penis vein, squares). Each administration group contained two rats. As an additional control 2 rats were intravenously injected with saline (black diamond). (a) Body weight of rats, measured on alternating days for one week after viral injections. (b) Viral infections were validated by Southern Blot analysis, using ^{32}P-labeled Apoptin gene as a probe. Lane 1-3 contain *Bam* HI digested pCMV-vp3 (5, 1 and 0.1 ng). Lane 4-7 contain liver-derived DNA from rats intravenously injected with AdMLPvp3 (lanes 4 and 5) or AdCMVluc (lanes 6 and 7). Exposure time for lanes 4-7 was approximately 10 times longer than for the control fragments.

For all administration routes also heart, lung, spleen, kidney, skin, testis and muscle samples were isolated for macroscopic evaluation and histological examination. No overt pathology was noted in these tissues, except for a slight increase in the mean spleen weight.

Also rats receiving a total of $8*10^9$ pfu AdMLPvp3 intravenously in 4 doses administered on two consecutive days, revealed no toxicity after 5 weeks (data not shown). The general parameters measured (including the weights of total body, spleen and liver), were similar to those of the control groups at the end of the experiment. From these data we conclude that significant amounts of the AdMLPvp3 adenovirus vector can be administered without acute fatal toxicity.

Discussion

This report describes the generation of a recombinant adenoviral vector expressing Apoptin and its effects on normal and on transformed cells. Our previous *in-vitro* studies[2, 3] have shown that Apoptin induces apoptosis in a range of human and rodent transformed and malignant cell lines, irrespective of p53, Bcl-2 or Bcr-abl status. We expected, therefore, that the generation and production of an adenovirus vector expressing the Apoptin gene might be hampered by the transformed nature of the helper cell lines. However, Apoptin-induced apoptosis in helper cells did not impede virus production. We previously showed that the 21-kD Ad-E1B protein[20,21]

partially inhibits Apoptin-induced apoptosis. Possibly, this protein somewhat delays the onset of apoptosis and therefore may facilitate propagation of adenovirus vectors that carry the Apoptin gene.

We report here that, after adenovirus mediated transfer of the Apoptin gene, human hepatoma cells are susceptible to Apoptin, whereas primary hepatocytes are not. Similar results with the *vp3*-containing vector were found for other combinations of cells, e.g. keratinocytes versus squamous cell carcinoma. In transformed cells, Apoptin is located in the nucleus and may interact with nucleic acids through its basic character. Due to its high proline content, the presence of Apoptin in the chromatin structure, may disturb the supercoiled organization resulting in DNA condensation and fragmentation. An alternative explanation might be that the basic and proline-rich character of the Apoptin protein enables it to act as a transcriptional regulator resulting in the induction of genes mediating apoptosis[22].

It appears that infection of HepG2 cells with an adenoviral vector encoding Apoptin leads to apoptosis faster than transfection with an expression vector. After DNA transfection, it takes approximately 3-4 days before 80% of the Apoptin-expressing transformed cells become apoptotic. In contrast, after adenovirus-mediated transfer of the Apoptin gene, this percentage is already reached after 24 hours (figure 3). Apparently, the adenoviral infection somehow stimulates Apoptin-induced apoptosis due to changes induced by the adenoviral infection, or, alternatively, the effective intracellular level of Apoptin is higher after viral infection than after transfection. Viral transduction does not, however, render normal cells sensitive to Apoptin.

The animal studies presented in this paper demonstrate a low toxicity of Apoptin *in vivo*, confirming and extending the results of the *in-vitro* studies. Rats intravenously injected with either the Apoptin- or luciferase encoding virus vectors showed a comparable increase of proliferating hepatocytes (an indirect measurement for liver damage). Also previous experiments with other adenoviral vectors studies revealed some increase in BrdU incorporation and the occurrence of apoptosis in these livers could be a response to the induction of the proliferative activity rather than a consequence of the transgene, especially since no difference is observed between the effects of the Apoptin- and the control vector.

In a first test for anti-tumor activity, AdMLPvp3 was injected into tumors arising from human hepatoma cells transplanted into nude mice. We observed a significant anti-tumor effect by Apoptin after a single intratumoral injection, despite the fact that this approach leads to transduction of only a part of the tumor. Given the finding that, *in vitro*, Apoptin-containing HepG2 cells are killed within a few days it may not be surprising that we do not find a high apoptotic activity in AdMLPvp3-treated tumors when evaluated 7 days after treatment. However, in (*pre*)clinical studies nowadays, repeated doses of a vector, e.g. Ad-p53, are administered [23-25]. It is therefore reasonable to anticipate that with AdMLPvp3, too, repeated injections will be even more effective.

In addition, Soruri et al. (1998) have shown that apoptotic tumor cells can trigger dendritic cells to process and present responding T lymphocytes, which will result in acquired cytotoxic responsive inducing apoptosis in the non-affected cells tumor cells[26]. If this mechanism also

holds true for Apoptin-induced apoptosis, its anti-tumor effect will be more pronounced in immune competent animals.

Southern blot analysis of the tumor material one week after injection with AdMLPvp3 showed that the viral DNA carrying the Apoptin gene was still detectable. The presence of the Apoptin gene after one week (figure 7C) may be due to infection of normal cells, e.g. stroma or endothelial cells.

The intrinsic tumor specificity of the Apoptin gene makes it a promising new tool for cancer gene-therapy. So far, attempts by others to obtain tumor-cell specificity rely on targeting the vector specifically to malignant cells, on tumor-specific expression of the transgene, or on local administration of the gene-transfer vehicle. To date, the applicability of the available systems is limited by their relatively low efficiency, or by lack of specificity[27-29]. Promising results have been obtained with the HSV-TK/gancyclovir combination in brain tumors[30], but applicability of this system outside the central nervous system (brain) is hampered by toxic effects for certain normal tissues, e.g. the cells of the liver parenchyma[31,32]. Another approach is based on the introduction of tumor-suppressor genes such as *p53* or the retinoblastoma protein (RB) into tumor cells in which the endogenous counterparts are affected[33, 34].

The p53 approach combines two important features for cancer therapy: efficacy and specificity. For instance, studies with Ad-p53 show that re-expression of *p53* in established tumors can induce apoptosis and tumor regression *in vivo*[35]. Both preclinical and clinical studies suggest a low toxicity for non-transformed cells when they are forced to express exogenous *p53* at levels sufficient for tumor-cell killing[23, 36,37]. However, the expression of wild-type *p53* is most effective in cells lacking functional p53. Tumor cells expressing wild-type *p53* are only marginally affected[35]. In vitro, at least, Apoptin does not seem to discriminate between $p53^-$ and $p53^+$ cells[2].

The tumor-cell specificity of Apoptin makes it a valuable new addition to the list of approaches that can be explored for cancer gene therapy. Although our preliminary *in-vivo* studies with AdMLPvp3 are promising, its potency and the more general applicability in other tumor cell types needs to be demonstrated.

Materials and Methods

Cells and cell culture

Ad5-*E1* (nt 459-3510)-transformed human embryonic retina (PER.C6)[13], Ad5- *E1* (nt 79-5789)-transformed human embryonic retina (911)[12], Ad5-transformed human embryonic kidney (293)[11] and human hepatoma (HepG2[38] and Hep3b[39])cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum (FCS) in a 5% CO_2 atmosphere at 37 °C. For immunofluorescence, cells were grown on glass microscope slides.

Primary rat hepatocytes were cultured in Williams E medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with insulin (2mU/ml) and dexamethasone (1 mM). The cells were grown on collagen-coated culture slides (Micronic, Lelystad, the Netherlands).

Plasmids and transfection

Expression plasmid pCMV-vp3 contains CAV DNA sequences encoding Apoptin (nt 427-868) under control of the cytomegalovirus promoter[2]. pCMV-desmin encodes desmin, a component of type II intermediate filaments, and was used as a negative control for the induction of apoptosis[40].

Plasmid pMad5[15] contains the *E1A* enhancer linked to the Major Late promoter (MLP) to drive expression of the transgene. Downstream of the transgene, the region of the Ad5 genome comprising nt 3328-8914 is located and is used for recombination with homologous sequences in pJM17[17]. After a *Bam*HI site was introduced into pMad5 to generate pMAD5/*Bam*HI, the coding region of Apoptin (nt 427-868) was inserted as a *Bam*HI fragment, in both orientations. The resulting plasmids were named pMad-vp3 and pMad-as. Plasmid DNA was purified by centrifugation in a CsCl gradient and column chromatography in Sephacryl S500 (Pharmacia, Uppsala, Sweden).

All transfections were performed by means of the calcium-phosphate precipitation procedure as described by Graham and Van der Eb[41]. Briefly, 2 hours prior to transfection the medium of 80% confluent monolayers of PER.C6, 911 or 293 was replaced by fresh medium. DNA-calcium-phosphate precipitates were incubated for 30 min at room temperature, and subsequently added to the medium. The cells were washed twice with phosphate-buffered saline (PBS) after 24 hours and fresh medium was added.

Immunofluorescence and DAPI staining

Indirect immunofluorescence was performed as described previously[42]. To demonstrate the presence of Apoptin and establish its cellular localization in transfected or infected cells, the cells were fixed with 80% acetone. The indirect immunofluorescence assay (IFA) was performed with a 100-fold dilution of the mouse monoclonal antibody (mAb) CVI-CAV-85.1 (85.1) for Apoptin[1], mAb 33 (Monosan, Uden, The Netherlands) for desmin and mAb lacZ (Boehringer Mannheim, The Netherlands) for ß-galactosidase. Fluorescein-isothiocyanate-labeled goat anti-mouse antibody (Jackson Immunoresearch Laboratories Inc., West Grove PA, USA) was used as second antibody. Nuclear DNA was stained with 1 μg/ml 2,4-diamino-2-phenylindole (DAPI) in 2% 1, 4 diazabicyclo-[2,2,2]-octane in glycerol/0.1 M TrisHCl pH 8.0[14].

Viruses and virus techniques

The recombinant adenoviral vector AdCMVlacZ carries the *E. coli LacZ* gene for β-galactosidase under control of the Cytomegalovirus enhancer/promoter. AdCMVluc contains the firefly *luciferase* gene under control of the CMV enhancer/promoter.

A recombinant adenoviral vector expressing Apoptin and a control vector were generated by cotransfecting near-confluent monolayers of 911 cells with equal amounts of pJM17[17] and pMad-vp3 or pMad-as (linearized by *Xmn*I digestion). After 24 hrs the medium was removed and the cells were washed twice with PBS. F15 minimal essential medium (MEM) containing 0.85% agarose (Sigma, USA), 20 mM HEPES pH 7.4, 12.3 mM MgCl2, 0.0025% L-glutamine, and 2% horse serum (HS) (heat-inactivated at 56°C for 30 min), was added. After 8-10 days, plaques were extracted and subjected to three rounds of plaque purification. The recombinant adenoviruses (rAdvs) were subsequently transferred to PER.C6 cells by a limited dilution assay.

Large-scale production of adenovirus was performed according to Fallaux et al.[12] Briefly, near-confluent PER.C6 monolayers in 600-ml flasks were infected with approximately 5 plaque-forming units per cell, in 8 ml DMEM containing 2% HS. After 2 hours at 37°C/5% CO_2, 12 ml DMEM/10%FCS was added to each flask. After 48 hr, the detaching cells were harvested and collected in PBS/2% HS. Virus was isolated from the producer cells by three cycles of freeze/thawing. The lysates were cleared by centrifugation at 2600 g for 5 min, and after dialysis against a sucrose-containing buffer, stored at -80°C.

Plaque assays were performed essentially as described by Graham and Prevec[43]. Briefly, adenovirus stocks were serially diluted in 1 ml DMEM/2% HS and added to near-confluent 911 cells in six-well plates. After 2 hours of incubation at 37°C/5% CO_2, the medium was replaced by agar-containing culture medium.

Quality control of rAdv batches

The expression of *Apoptin* by the adenoviral vectors after infection of helper cells was tested by indirect immunofluorescence as described. The rAdV stocks were screened for the presence of replication-competent adenoviruses (RCA) by a polymerase chain reaction (PCR) assay. A 615-bp region was amplified with one oligonucleotide directed to the ITR of the adenoviral DNA (5'GGGTGGAGTTTGTGACGTG-3') present in the Ad vector, and one to the region coding for the E1A protein (5'TCGTGAAGGGTAGGTGGTTC-3'), which is not present in the vector. The presence of the 615 bp product is indicative for the presence of RCA.

In brief, adenovirus supernatant (3* freezed/thawed) was degraded by digestion with proteinase K . After boiling, 5 μl of the inactivated virus was added to 45 μl of 50 mM KCl, 10 mM TrisHCl (pH 8.4), 0.1 mg/ml gelatine, 0.05%

Tween20, 1.75 mM $MgCl_2$, 0.1 mM of each deoxyribonucleotide, 20 pmol forward primer, 20 pmol reverse primer, 250 ng tRNA and 3 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, USA). PCR is performed for 30 cycles (94 °C 1 min, 63 °C 2 min, 72 °C 3 min) in an automated DNA Thermal Cycler (Perkin-Elmer Cetus). DNA isolated from 911 cells infected with a pseudo-wild-type adenovirus (*dl*7001) served as a positive control. The assay has been optimised and detects 1 pfu of an *E1*-containing adenovirus amidst 10^7pfu of *E1*-deleted vector (Van den Wollenberg & Hoeben, unpublished results).

TUNEL assay

Tdt-mediated dUTP nick end labeling (TUNEL) was performed with the use of the in situ cell death detection kit, fluorescein (Boehringer Mannheim). Forty hours after transfection cells were washed with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4), for 30 minutes at room temperature. After permeabilisation (0.1% Triton X-100, 0.1% sodium citrate, 2 minutes at 4°C) cells were incubated with the TUNEL reaction mixture (containing fluorescein labeled nucleotide polymers and terminal deoxynucleotidyl transferase) for one hour at 37°C. After washing with PBS, the cells were analysed by fluorescence microscopy.

Giemsa staining and β-galactosidase assays

For detection of the number of attached cells, cells were washed twice with PBS and fixed in methanol/acetic acid (3:1) for 15 minutes at room temperature. For 30 minutes, cells were incubated with Giemsa solution (3% Giemsa (Merck, Darmstad, Germany) in 1 mM Na_2HPO_4, pH 7.0) at room temperature. After staining, the cells were washed four times with deionized water and allowed to dry by air.

For detection of *LacZ*-encoded β-galactosidase activity, cells in tissue culture were fixed 48 hours after infection in ice-cold 2% paraformaldehyde/0.2% glutaraldehyde solution, washed in ice-cold PBS (containing 2 mM $MgCl_2$), and incubated in 3 ml of reaction mix (1 mg/ml X-Gal (Boehringer, Mannheim, Germany), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS) at 37°C for 4-16 hours[44].

Animals

Male Balb/$c^{nu/nu}$ mice, aged 7-8 weeks, and male Wag/Rij rats, weighing 180-210 g were obtained from Harlan (Zeist, The Netherlands). Animals were kept at standard laboratory conditions of alternating 12-h periods of light and darkness and a standard laboratory diet. The animal experiments were approved by the Leiden University Animal Welfare Committee.

Histology

Tissue sections were fixed in 4% buffered formalin. After paraffin-embedding, sectioning (ca 4 micron) and mounting on slides, tissue sections were routinely stained with hematoxylin-eosin (HE).

In the toxicity experiment,rats were injected intraperitoneally with 50 mg/kg bromodeoxyuridine (BrdU, Sigma) in saline, two hours prior to sacrifice. DNA-incorporated BrdU, as a measurement for cellular DNA replication, was detected immunohistochemically by a three-step immunoperoxidase staining with the anti-BrdU monoclonal antibody IU_4[45]. Sections were counterstained with haematoxylin. Both HE and BrdU/H stained rat liver sections were analysed using a Zeiss Axioplot microscope (Dena, Germany) and a 40x objective. In HE stained sections both apoptotic cells – characterized by dense, often fragmented, nuclei, eosinophilic cytoplasm and reduced contact with neighbour cells- and mitotic cells –defined by the absence of nuclear morphology, but the appearance of chromosomes in meta-, ana- or telophase- were counted. In anti-BrdU sections we discriminated BrdU-positive liver parenchymal cells (relatively large round nuclei) from (all) other cell types in the liver. For each parameter, 100 randomly distributed areas were scored in each liver section (mostly containing 3-4 lobes) and each area contained approximately 150 nuclei.

For detection of AdCMVlacZ encoded β-galactosidase, paraffin sections were stained with the polyclonal rabbit antibody 55978 (Cappel, Aurora, Ohio, USA). After deparaffinisation, slides were incubated with the anti-β galactosidase antibody (55978, 1:2000) for 1 hour at room temperature. As a second antibody biotinylated swine anti-rabbit (Dako, 1:300) was used, followed by streptavidine-conjugated peroxidase. Cells were rinsed with Trizma (Sigma) and positive cells were revealed by 15 minute exposure to Dab solution in Trizma.

Southern-blot analysis

DNA was isolated from isopentane-fixed tissue and digested with *Bam*HI. Southern[46] analyses were performed, by DNA fractionation on a 1% agarose gel and blotting onto Hybond$^+$ (Amersham, UK). The blots were hybridized with randomly primed ^{32}P-labeled DNA fragment encoding Apoptin. In all assays *Bam*HI digested pCMV-vp3 plasmid DNA was used as a control. The signals were quantitated by phosphor imaging.

Acknowledgments

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Chapter 3

Gene therapy with Apoptin induces regression of xenografted human hepatomas

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Abstract

The Chicken Anaemia Virus (CAV)-derived Apoptin protein shows remarkable specificity; namely, it induces apoptosis in tumour cells, but not in normal diploid cells. We have exploited the Apoptin gene for use in cancer gene therapy. Here we demonstrate that adenovirus-mediated intratumoural transfer and expression of the Apoptin gene results in regression or complete remission of human hepatomas grown as xenografts in immune-deficient mice, and significantly increases their survival long-term. Early after intratumoural injection, Apoptin could be detected in significant quantities by Western-blot analyses and immunohistochemistry. Furthermore, cell death and disruption of the tumour integrity were apparent in the transduced regions. This experimental gene therapeutic strategy constitutes a unique example of specific anti-tumour activity using a virus-derived gene with broad-spectrum applicability.

Introduction

Current anti-cancer therapies are limited by toxicity to normal tissue and the occurrence of therapy-resistant tumour cells. The Apoptin protein, encoded by the Chicken Anaemia Virus (CAV), shows unexpected specificity and potency towards human tumour cells. In young chickens, CAV infection leads to a depletion of the thymus caused by extensive apoptosis.[1,2] In transfection studies, Apoptin was shown to induce apoptosis in a large panel of transformed and malignant cells of avian, murine, and human origin including carcinomas, sarcomas, melanoma, lymphoma and leukaemia.[3,4]

Remarkably, Apoptin did not induce apoptosis in 'normal', diploid cells.[4] However, transformation of diploid human fibroblasts or keratinocytes by expression of SV40 large-T antigen rendered these cells sensitive to Apoptin-induced apoptosis.[5] The apoptosis-inducing activity correlates with the subcellular localisation of the protein. Specifically, in normal cells Apoptin resides predominantly in the cytoplasm, whereas in transformed cells, it localises to the nucleus.[3]

Intriguingly, although Apoptin-induced apoptosis involves caspase-3, it bypasses most of the upstream components of the apoptotic pathway, making it resistant to mutations in this pathway.[6] Moreover, Apoptin-induced apoptosis is not affected by loss of functional p53, or by over-expression of either Bcl-2 or Bcr-abl, conditions that often frustrate conventional therapies such as chemotherapy and radiation. Indeed, it is even stimulated by Bcl-2 overexpression.[7] Thus, in addition to its intrinsic specificity, which allows selective action against transformed cells, Apoptin may also work in cases when chemotherapy and radiation have failed. Although the mechanism by which Apoptin distinguishes malignant cells from their 'normal' primary counterparts has not yet been fully elucidated, it is attractive to exploit these abilities for cancer gene therapy.

To examine the anti-tumour effect of Apoptin, we generated a replication-deficient adenovirus containing the Apoptin gene (AdMLPvp3, further referred to as AdMLP.Apoptin). We showed that adenoviral expression of Apoptin both in vitro and in vivo did not change its specificity; namely, it still induced apoptosis in tumour cells and had no deleterious effects on normal cells. The first, short-term pilot experiment, which comprised a single intratumoural injection of AdMLP.Apoptin, the adenovirus vector that carries the Apoptin gene, in subcutaneous human hepatoma in nude mice, showed a significant decrease in tumour growth.[8] Because we noticed that a single intratumoural injection only reaches a fraction of the tumour cells, we designed a regimen using multiple injections to determine whether AdMLP.Apoptin can cause actual tumour regression. A parallel experiment was carried out to follow the effects of AdMLP.Apoptin at the cellular level.

In this study we show the induction of apoptosis by AdMLP.Apoptin in HepG2 (human hepatoma) tumours similar to that observed for CAV in transformed chicken cells.[9] Furthermore, multiple injections of the adenovirus vector with the Apoptin gene resulted in partial or complete regression of the established tumours in the majority of the recipient mice. Our data show for the first time that treatment with Apoptin significantly improves long-term survival of tumour-bearing animals.

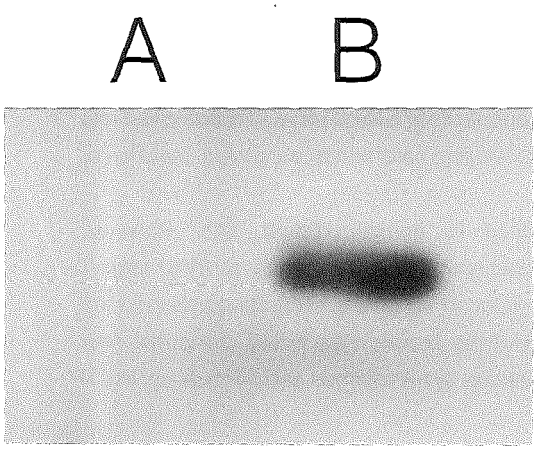


Figure 1. Apoptin expression after injection of AdMLP.Apoptin in HepG2 tumours.

Two days after intratumoural injection of buffer (lane A) or AdMLP.Apoptin (lane B), HepG2 tumours were lysed and the protein fractions were subjected to immunoprecipitation with a polyclonal rabbit antibody specific for the C-terminus of Apoptin. After blotting, the proteins were immunoprobed with mAb 111.3, which is directed against the N-terminus of Apoptin.

Results

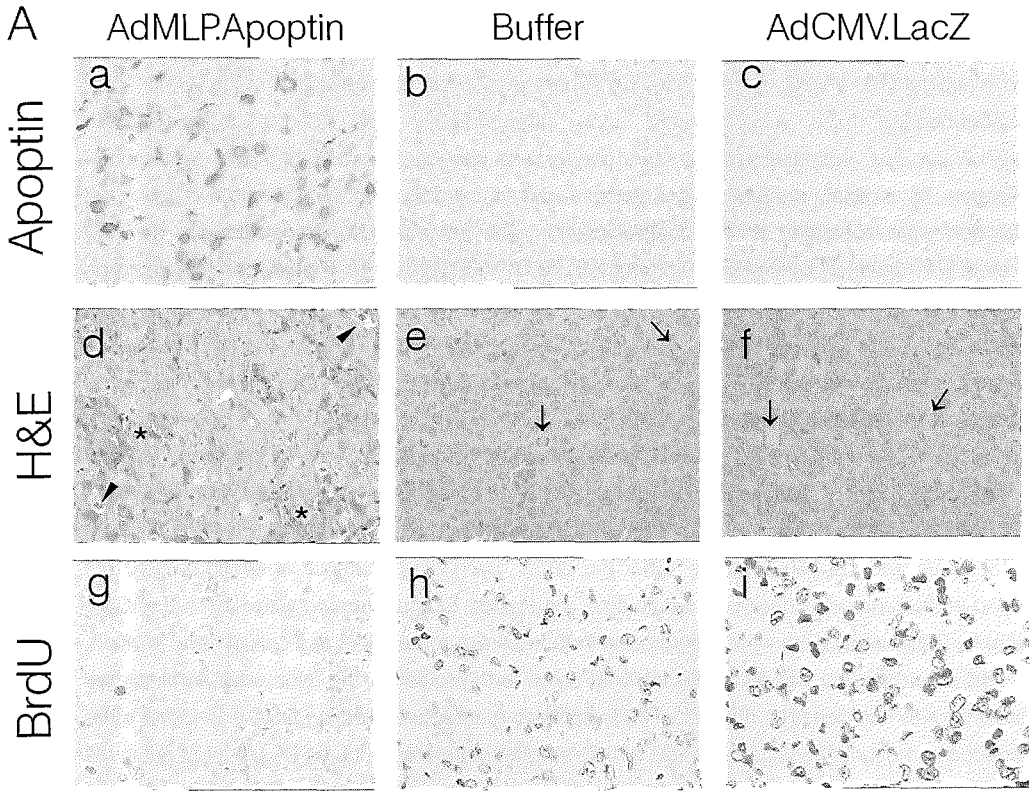
To investigate the effects of the adenovirus expressing Apoptin on a cellular level in vivo, HepG2 tumour-bearing mice were injected once intratumourally with AdMLP.Apoptin or with AdCMV.LacZ or virus dilution buffer as a control, and were sacrificed 2,3,4,5 and 6 days post-injection. To ascertain the presence of Apoptin, protein extracts from the tumours two days post injection were subjected to immunoprecipitation with an antibody against Apoptin and the protein was detected by Western Blot analysis (fig 1). Additionally, the Apoptin protein could be readily detected in HepG2 tumour tissue sections by immunohistochemistry using the polyclonal antibody anti-VP3C (fig 2A,a). In contrast, no Apoptin signal was detected in the AdCMV.LacZ or buffer control (fig 2A,b,c). All stainings were performed on serial sections to determine the areas of the tumour transduced with adenovirus.

Tumour areas expressing Apoptin exhibited aberrant morphology characterised by loss of tissue integrity, increase in interstitial space and visible remnants of disintegrated cells. These disrupted areas showed loss of cell-cell contact and tumour cells containing hypodense cytoplasms and condensed dark nuclei (fig 2A,d). Such morphological aberrations were not found in the β-galactosidase-positive fields of the HepG2 tumours treated with AdCMV.LacZ (fig 2A,e,f). These data demonstrate an Apoptin-induced anti-tumour effect at a cellular level in vivo.

To determine the viability of the infected areas in the tumours, the mice were administered BrdU prior to sacrifice. The number of cells containing actively-replicating DNA was found to be reduced in most of the disrupted areas of the Apoptin-treated tumours (fig 2A,g,h,i) but not in the β-galactosidase-positive areas (β-galactosidase staining not shown). This suggests that Apoptin-transduced areas are repressed in their outgrowth.

Over time, the number of Apoptin-positive cells decreased within the HepG2 tumours. Two days post-injection, several areas of the tumours showed up to 40% Apoptin-positive cells, as determined by immunohistochemistry (fig 2B,a). On subsequent days, however, the number of Apoptin-positive cells decreased to less than 1% (fig 2B,b,c). Between day 2 and 6, the Apoptin pattern distinctly changed: from a mixture of cells with homogeneously distributed nuclear and some cytoplasmic staining, to exclusively condensed nuclear staining. After six days, the Apoptin-positive cells had almost completely disappeared and only sporadic fragments of positive cells were found (fig 2B,c). ß-galactosidase-expressing cells in tumours injected with AdCMV.LacZ, however, were still abundant after six days. In contrast to Apoptin-positive cells, virus-infected cells expressing ß-galactosidase appeared histologically normal (fig 2B,d,e,f), indicating a toxic effect of Apoptin, but not ß-galactosidase, on the HepG2 cells.

Light-microscopic evaluation of individual Apoptin-positive cells at high magnification revealed a typical pattern of the Apoptin distribution within the nuclei. Two days after infection, the distribution was faint and finely granular. Later the granules increased in size, and gradually accumulated into "doughnut" shaped structures (fig 3a). Strikingly, these distribution patterns of Apoptin within the tumour cell nuclei closely resembled those observed for CAV-infected transformed chicken lymphoblastoid cells (fig 3b).[9] Apparently, the effects of Apoptin are very similar when delivered by an adenovirus or by its natural vector, the chicken anaemia virus.



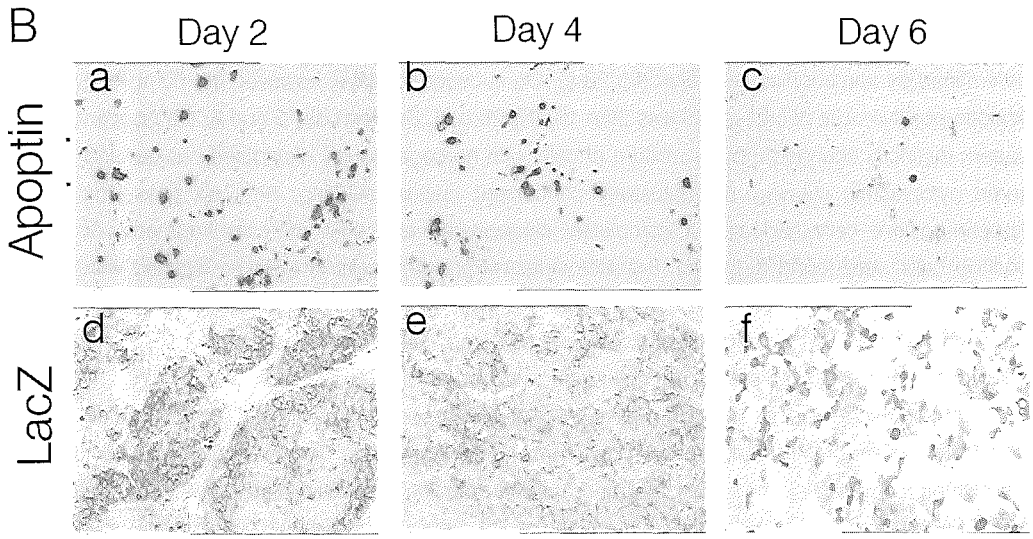


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Figure 2 , Effects of Apoptin gene therapy on HepG2 tumours at the cellular level.

A Tumour-bearing mice were treated with a single intratumoural injection of $5*10^9$ pfu AdMLP.Apoptin, AdCMV.LacZ or were injected with virus dilution buffer. At day 2,3,4,5 and 6 after treatment, four mice per treatment group were sacrificed and tumours were processed for immunohistological analyses. Depicted are paraffin sections of tumours fixed two days after treatment. *a, b,c,* Sections were incubated with an Apoptin-specific antibody and labelled with peroxidase-coupled second antibody. Cells expressing Apoptin are visualised by DAB signal. The Apoptin protein was readily and exclusively detected in AdMLP.Apoptin-treated animals *(a).* In buffer *(b)* and AdCMV.LacZ-treated animals *(c),* no Apoptin was detected. *d, e, f,* Haematoxylin/eosin staining of sequential sections of a, b and c. *(d)* At the tumour sites infected with AdMLP.Apoptin, tumour cell integrity was disrupted with decreased cell-cell contact (▷), containing enlarged hypodense cells with dark shrunken nuclei. Scattered throughout these areas, fragments of disintegrated cells (∗) and apoptotic cells (►) were observed. In contrast, tumours treated with buffer *(e)* and AdCMV.LacZ *(f)* showed a uniform and regular structure. Additionally, frequent mitotic figures were present (arrows). *g, h, i,* Mice were injected with BrdU prior to sacrifice, to determine the number of DNA-replicating cells in the HepG2 tumours. *(g)* Anti-BrdU-staining in areas of Apoptin expression (sequential sections) revealed predominantly low incorporation of label. In contrast, areas expressing ß-galactosidase *(h)* or injected with buffer *(i)* showed persistent BrdU-incorporation.

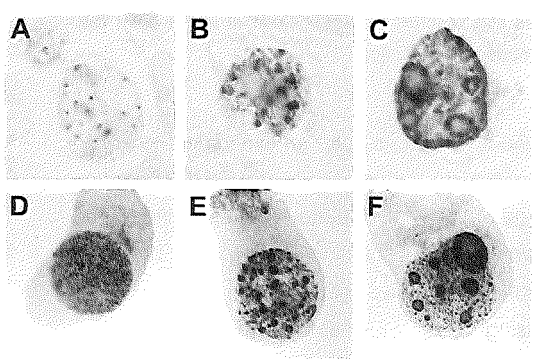
Figure 2 B, Effects of Apoptin gene therapy on HepG2 tumours at the cellular level.

B, Transgene expression over time after recombinant adenovirus injection: sections of HepG2 tumours growing in nude mice. The animals were sacrificed 2, 4 or 6 days after a single virus injection (AdMLP.Apoptin or AdCMV.LacZ). Upper panel: Apoptin staining as described under fig2A. Lower panel; ß-galactosidase expression in AdCMV.LacZ-treated tumours was determined by labelling with a specific antibody, visualised by DAB oxidation.

a, b, c, high expression of Apoptin is detected in tumours two days after intratumoural AdMLP.Apoptin injection. After four days the number of Apoptin-positive cells decreases and after six days only sporadic Apoptin expression is found. Furthermore, the structure of Apoptin staining in the cells also changes over time. Early after virus-infection the tumour cells show both cytoplasmic and nuclear localised Apoptin (*a*). Later, mostly nuclei are stained positive (*b, c*). Six days after injection, predominantly fragments of stained tumour cells and few intact stained tumour cells are detected (*c*). *d, e, f,* The number of ß-galactosidase-expressing cells in AdCMV.LacZ-injected tumours remains constant over time. Two days after infection a high number of positive cells was detected *(d)*. In contrast to the Apoptin expression in Apoptin-treated animals, ß-galactosidase is still present in a high percentage of cells four (*e*) and six (*f*) days after virus injection.

Figure 3 Immunoperoxidase staining of Apoptin.

A, B, C, Staining on paraffin sections of xenografted human HepG2 tumours infected with adenovirus expressing the Apoptin protein with Apoptin-specific antibody 111.3. The tissue sections were fixed and stained 2 days after infection; three representative images are shown (original magnification: x 1,250). Typically, early after infection, the distribution was faint and finely granular *(A)*. Later the granules increased in size *(B)*, and gradually accumulated into "doughnut" shaped structures *(C)*. Strikingly, these distribution patterns of Apoptin within the tumour cell nuclei closely resembled those observed for CAV-infected transformed chicken lymphoblastoid cells [9].



D, E, F, For comparison purpose, indirect immunoperoxidase staining of CAV-infected chicken lymphoblastoid T cells is shown from Noteborn *et al* (1994)[9]. In this experiment the apoptin-specific antibody 85.1 was used and the cells were fixed and stained 76 hours after infection.

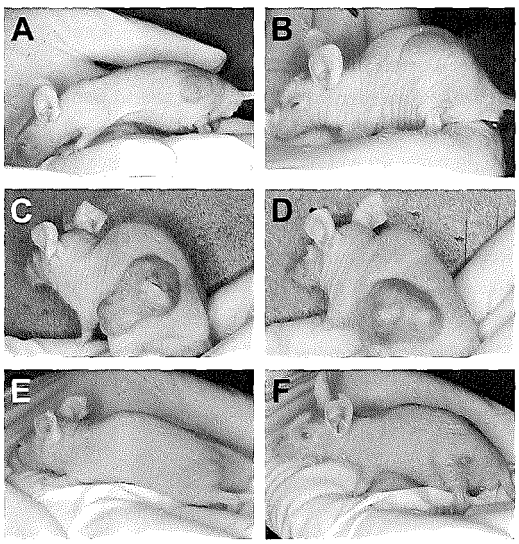


Figure 4 Human hepatomas (HepG2) in nude mice before and after Apoptin gene therapy treatment.

Subcutanously grown HepG2 tumours were injected five times on alternating days with $3*10^9$ pfu AdMLP.Apoptin, $3*10^9$ pfu AdCMV.LacZ or buffer. Hereafter, tumour growth was measured regularly. *(a)* and *(b)*, examples of HepG2 tumours at the start of treatment. The criteria to start virus-treatment were met at this point: a tumour volume > 250 mm^3 *and* tumour height of 3,0 mm. *(c)* and *(d)*, examples of HepG2 tumours treated with LacZ or buffer at the point when the criteria were met to end the experiment. The end-criteria were: a tumour volume of > 1800 mm^3 *and* a tumour height of 9 mm. *(e)* and *(f)*, tumour regression upon AdMLP.Apoptin treatment. Examples of partial and complete remission *(e, f)* of HepG2 tumours achieved after Apoptin-gene therapy.

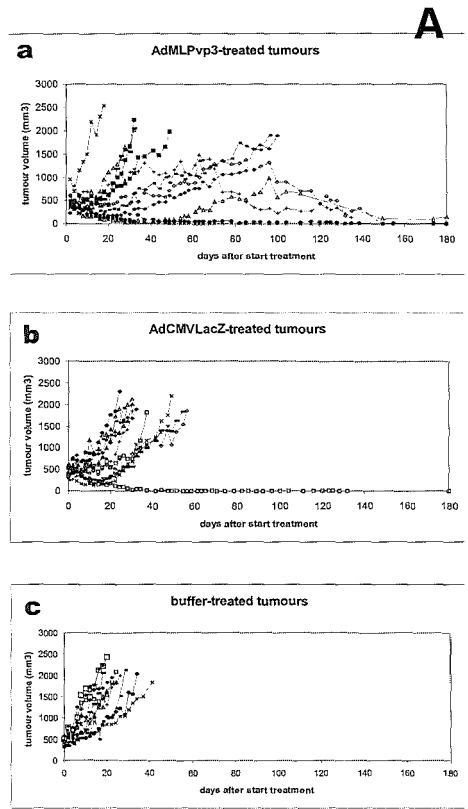
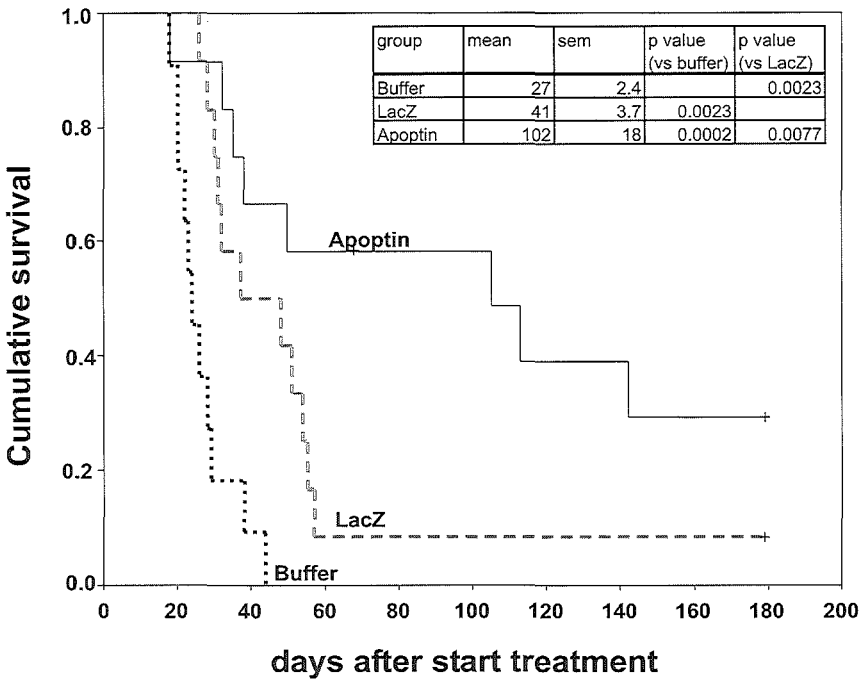


Figure 5A Long-term effect of Apoptin gene therapy in vivo. *Tumour growth kinetics after treatment*

Nude mice (nu/nu) were implanted subcutaneously with HepG2 cells and tumour growth was determined every other day. Each individual mouse was monitored until the criteria were met to enter the experiment. At that point each mouse was randomly divided for multiple injections either with AdMLP.Apoptin *(a)*, AdCMV.LacZ *(b)* or buffer *(c)*. In the following weeks each tumour was measured until its size reached the end-criteria at which point the mouse was sacrificed.

To further evaluate the potential of Apoptin gene therapy, we designed an experiment to study the long-term effects and survival benefit of treating tumour-bearing mice with AdMLP.Apoptin. Based on previous observations that the percentage of transduced tumour cells is limited after one intratumoural injection, we attempted to achieve overall transduction of the tumour by using an improved treatment protocol. This protocol consisted of five intratumoural injections per tumour every other day, each injection comprising $3*10^9$ pfu AdMLP.Apoptin, $3*10^9$ pfu



group	mean	sem	p value (vs buffer)	p value (vs LacZ)
Buffer	27	2.4		0.0023
LacZ	41	3.7	0.0023	
Apoptin	102	18	0.0002	0.0077

Figure 5B Long-term effect of Apoptin gene therapy in vivo. *Survival analysis*

Mice treated with AdMLP.Apoptin survived longer than the mice in the other two groups ($p<0.01$). One hundred days after the beginning of the treatment still 60% of the animals treated with AdMLP.Apoptin (straight line) were alive, whereas at that point 90% of AdCMV.LacZ (dashed grey line) and 100% of buffer-treated (dotted black line) mice had met the end-criteria and were sacrificed by the experimentator. *Insert* Mean survival after Apoptin gene therapy. Tumour-bearing mice treated with buffer had a mean survival of 27 days (standard error of the mean = 2.4). The mean survival for AdCMV.LacZ was 41 days (sem = 3.7). The mean survival of Apoptin-treated animals was almost twice as long compared to LacZ and three times longer than the buffer-treated mice.

AdCMV.LacZ or sucrose buffer. At the time of treatment the tumours had a volume of minimally 250 mm^3 and a tumour height of at least 3 mm (fig 4a,b). Each tumour was measured four times weekly. The virus-injected tumours showed a pale appearance during the 10-day treatment period. In order to optimise the treatment, the inoculation was administered to the areas of the tumour that appeared most viable, as judged by the dark-red colour of non-treated HepG2 tumours. After the treatment period, the AdCMV.LacZ-treated tumours gradually regained this dark-red colour, whereas Apoptin-treated tumours remained pale.

The growth kinetics of the tumours following treatment are shown in figure 5A. During treatment, the tumours in both virus groups were delayed in growth, but soon after the end of treatment, the LacZ-treated tumours resumed growing (fig 5A,b) whereas most of the Apoptin-treated tumours did not (fig 5A,a). The majority of the mice treated with AdMLP.Apoptin showed partial or complete response to treatment (fig 4e,f). However, there were also several non-responders to

the Apoptin-treatment, which had to be sacrificed early in the experiment along with animals from the buffer- and AdCMV.LacZ treated groups (fig 5A a,b,c). Animals were sacrificed when their tumour had reached a minimal size of 1800 mm^3 and a height of at least 8 mm (fig 4c,d).

Between 8 and 28 days after intratumoral injection, all buffer-injected animals had to be sacrificed. After 60 days, only 1 of 12 of the AdCMV.LacZ-treated mice survived, whereas at that point 7/12 of Apoptin-treated animals were still alive (fig 5B). To comply with the standard criteria for normal distribution, one outlier from the LacZ-treated group was excluded from statistical analysis. This results in a mean survival of LacZ-treated mice of 41 days, compared to a mean survival of buffer-treated mice of 27 days (fig 5B). Tumour-bearing mice treated with AdMLP.Apoptin, however, survived much longer than the mice in both other groups (mean survival 102 days; $p<0.01$). Six months after treatment, 30% of Apoptin-treated animals were completely tumour-free. These results demonstrate that Ad-Apoptin can confer significant survival benefits and tumour reduction when used in vivo.

Discussion

Here, we describe an unconventional approach for cancer gene therapy that is based on the tumour-specific activity of Apoptin. We initially observed that adenoviral transfer of Apoptin into subcutaneous HepG2 tumours in nude mice had a negative effect on tumour growth.[8] To further investigate the therapeutic benefit of AdMLP.Apoptin for these tumours, we now treated well-established tumours with multiple injections over a period of 10 days. With this approach we were able to achieve complete regression of tumours treated with AdMLP.Apoptin, thus providing proof of principle that Apoptin can be used as an anti-cancer agent.

Regarded in more detail, the Apoptin-treated tumours can be divided into three distinct groups: those with a complete response, those with a significant delay in tumour growth, and those with tumour growth kinetics similar to tumours treated with AdCMV.LacZ. Because no replicating virus is produced, any tumour cell that escapes viral infection during the course of the five treatments will continue to proliferate, provided that there is not too much surrounding damage. Thus, differences in response to Apoptin treatment are probably an effect of adenoviral dispersion through the tumour. Indeed, the characteristic architecture of HepG2 tumours, with their partitioned lobular structure, does not allow an even distribution throughout the tumour after a single injection.[8] Thus, it is plausible that complete regression occurred only in tumours in which all lobes received sufficient adenovirus. The partially responding tumours were most likely only transduced in certain areas; although substantial tumour cell death and disruption of tissue integrity contributed to a delayed outgrowth of these HepG2 tumours, there was not enough viral dispersion to completely eliminate the tumour. In the case of the non-responders, probably only a minor percentage of tumour cells were infected, resulting in a rapid outgrowth of the non-transduced cells. Nevertheless, this in vivo experiment shows a significant survival benefit for tumour-bearing mice treated with AdMLP.Apoptin, provided that a substantial percentage of tumour cells can be transduced with Apoptin.

Investigation of the anti-tumour effect of Apoptin at the cellular level shows that a single AdMLP.Apoptin injection causes substantial damage to HepG2 tumours. In contrast, control-treated tumours showed normal morphology and high proliferation, as determined by BrdU labelling. The areas of AdMLP.Apoptin-treated tumours containing Apoptin-positive cells, however, showed extensive aberrant morphology and a substantial decrease in proliferating cells, already detectable at two days after injection. The extent of damage to the tumour architecture surpassed that which would be expected based on the number of positive cells that was detected. This observation likely indicates a deleterious effect of dying Apoptin-positive cells on surrounding, non-infected cells. Thus, in addition to specific tumour cell-killing by Apoptin, it is possible that a critical threshold of apoptosis in tissue can lead to a bystander effect on non-transduced cells, thereby increasing the overall efficacy.

There are several pieces of evidence suggesting that AdMLP.Apoptin induced apoptosis in the treated tumours. Firstly, Apoptin accumulates in HepG2 tumours in similar structures that arise when apoptosis is induced by CAV in chicken lymphoblastoid cells. Secondly, Apoptin induces rapid apoptosis in HepG2 cells in vitro.[8] Finally, in contrast to LacZ-transduced tumours, Apoptin-positive cells are nearly depleted 6 days after injection. Taken together, these findings strongly suggest that the damage observed in these HepG2 tumours is a consequence of Apoptin-induced apoptosis. Although Haematoxylin and Eosin staining showed a trend towards increased apoptosis in tumours treated with AdMLP.Apoptin, examination of DNA fragmentation by TUNEL staining could not confirm this observation (data not shown). This difficulty in detecting apoptosis in vivo has been described for several in vivo apoptosis systems; for instance, the complete involution of islets of Langerhans in myc-transgenic mice[10] or the disappearance of the prostate in castrated rats[11]. In these cases, the apoptotic process combined with phagocytosis occur in such a short time span[12-14], that only occasional apoptotic cells are detected. Nevertheless, the entire tissue eventually disappears.

Gene therapy with Apoptin offers unique advantages over current approaches for cancer therapy. For example, resistance to cancer therapies is often caused by the inactivation of apoptotic pathways, which has occurred in the majority of tumours.[15-17] Gene therapeutic approaches based on restoring part of the apoptotic pathway, for instance transduction of p53, p16, Bax and Bcl-x, are expected to be successful in a limited subset of tumours, depending on the particular mutations in their apoptotic machinery. The fact that Apoptin does not need a functional p53 pathway, is not hindered by common blockage of apoptosis by Bcl-2 or Bcr-abl expression and apparently acts downstream of most decision factors, suggest it will be applicable to a wide range of tumours. In vitro, Apoptin induces apoptosis in every cancer cell type of the extensive panel that has been tested.[18]

In addition to therapy-resistance, toxicity to normal tissues often hampers cancer therapies such as chemotherapy and radiation. Moreover, most conventional gene therapeutic approaches suffer from insufficient tumour cell specificity, as illustrated by reports on toxic effects in healthy tissues due to the use of the Herpes Simplex Virus Thymidine Kinase (HSV-TK).[19,20] Attempts to increase selectivity further include the use of tumour-specific promoters (e.g.

CEA, AFP) or targeted vectors. Even if the desired specificity does not abolish the necessary potency, these strategies, again, are likely to be applicable to only a subset of tumour types. In marked contrast, extensive in vitro data show that Apoptin has unparalleled specificity; despite its potency towards tumour cells, no normal cell type tested thus far has ever shown sensitivity to Apoptin.[3,4,18] Consistent with this, during the in vivo experiments described here, we did not observe any toxic effects of AdMLP.Apoptin treatment, corroborating the data of a more extensive toxicity study after intravenous injection of AdMLP.Apoptin in healthy rats.[8]

The combination of potency and specificity residing in a single molecule provides new possibilities for cancer therapy. So far, the therapeutic use of Apoptin seems to be predominantly limited by factors in common with most gene therapy strategies; namely, the ability to transduce all target cells. New delivery methods are being developed to increase efficacy of spread throughout solid tumors and throughout the body. The Apoptin gene is exquisitely suited for inclusion in technologies such as conditionally replicative viruses[21,22] or non-viral transduction methods due to its potency and small size. However, these new delivery strategies still have to be validated. At this time, the marked anti-tumour effect and survival benefits of Ad-Apoptin as we have shown in this paper already warrant its evaluation for human clinical trials in the near future.

Acknowledgements

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Methods

Cells and cell culture.

Human hepatoma cells (HepG2) (obtained from ATCC, USA), mycoplasm free and negative in Mouse antibody Production (MAP) test, were grown in Dulbeco's modified Eagle's medium (DMEM) CO_2 (GIBCO laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) at 37°C/5%. The cells were harvested by trypsinization, resuspended in cold Hanks (13.6 mM NaCl, 530 μM KCl, 81 μM $MgSO_4$, 44 μM KH_2PO_4 and 34 μM Na_2HPO_4) containing 2% horse serum and the viable cell number was determined by trypan blue exclusion and adjusted to $5*10^7$ cells/ml in serum-free Hanks. Within one hour of harvesting, the cells were injected subcutaneously in the flanks of nude mice, depositing 200 μl cell suspension per flank with a 25-gauge needle. The cells were kept on ice during the time between harvest and injection.

Viruses and virus techniques.

The recombinant adenovirus vector expressing Apoptin (Ad.MLP.vp3, further referred to as AdMLP.Apoptin) was generated as described previously.[8] Briefly, 911 helper cells were cotransfected with the linearized Ad adapter construct, pMad-vp3, and pJM17. After homologous recombination, this results in an Ad5 genome lacking the E1 and E3 regions, containing the transgene under control of the adenovirus major late promoter (MLP). Plaques were isolated and after three rounds of plaque purification transferred to PER.c6 cells. These cells contain the E1 region regulated by a heterologous promoter which, combined with matched adaptor plasmids, eliminates the generation of RCA by heterologous recombination.[23] All batches of AdMLP.Apoptin were tested for Apoptin expression by indirect

immunofluorescence [24] and for the presence of RCA by a PCR assay.[8] All virus batches used passed the RCA test detecting 1 pfu of an E1-containing adenovirus amidst 10^7 pfu of an E1-deleted vector. Construction of the recombinant adenovirus vector AdCMV.LacZ under the control of the cytomegalovirus enhancer/promoter has been reported previously.[25] Recombinant viruses were propagated on PER.c6 cells and purified by double CsCl density centrifugation. Titers of the viral stocks were determined both by plaque assay on 911 cells.[26] Virus was alliquotted and stored in sucrose buffer (140 mM NaCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 20 mM $MgCl_2$ and 5% sucrose) at - 80°C.

Immunoblot.

After sacrifice, tumours were dissected and half of each tumour was fixed in 4% formaldehyde for immunohistochemistry and half was flash-frozen in –80 °C cold isopentane. Frozen samples were subsequently grinded with a microdismembrator, resuspended in 1ml lysis buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.1% Triton and protease inhibitors) and incubated on ice for one hour. The lysate was cleared by centrifugation and immunoprecipitated with protA beads (Santa Cruz biotechnology, Santa Cruz, California, USA) coupled to a polyclonal rabbit antibody recognising the C-terminus of Apoptin, αVP3C. The immunoprecipitates were washed three times with cold lysis buffer and boiled for 5 min in Laemli sample buffer. Samples were subjected to electrophoresis on a 12.5% polyacrylamide SDS gel, followed by transfer to Immobilon P membranes (Millipore, Bedford, Massachusetts, USA). After blocking in milk buffer (5% milk in Tris-buffered saline with 0.2% Tween (TBST)) the membranes were incubated with the mouse monoclonal antibody 111.3 against the N-terminus of Apoptin in milk buffer overnight at 4 °C. The membranes were washed with TBST, and proteins were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, Zwijndrecht, The Netherlands) secondary antibody, followed by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, New Jersey, USA).

Animals.

Male Balb/c nu/nu mice, aged 7-8 weeks (Harlan, The Netherlands) were kept in filtertop cages under DII-safety conditions following Dutch government guide lines. Animals were fed sterilised laboratory chow and water ad libitum and were kept at alternating 12-hour periods of light and darkness. All experiments were approved in advance by the Dutch animal welfare committee.

Evaluation of cellular effects after a single intratumoral injection of HepG2 tumours with AdMLP.Apoptin.

Mice were injected subcutaneously (s.c.) in both flanks with $1*10^7$ HepG2 cells suspended in 200µl serum-free Hanks. The mice were checked daily for tumour growth. Once a tumour became detectable by eye, registration of tumour growth started by measuring tumour length, width and height using microcalipers and tumour volume was calculated every other day. Calculation of tumour volume was done using the following formula: [smallest diameter^2]*[largest diameter]/2. Three weeks after tumour cell injection, when tumours had reached an average volume of 350mm^3, the mice were randomised for one of the three treatment groups (AdMLP.Apoptin, AdCMV.LacZ or virus dilution buffer), and each tumour received an intratumoural injection with $5*10^9$ pfu virus or control buffer in a total volume of 100 µl. Each following day all mice were weighed and tumours were measured and examined for changes in colour or morphology. At day 2, 3, 4, 5 and 6 days after intratumoural injection a group of mice was sacrificed, comprising of 3-4 tumours of each treatment group. At sacrifice, blood was collected, and after removal, the tumours, liver and spleen were weighed and stored partly in 3,7% formalin or were flash-frozen in liquid nitrogen for the prospective measurements. One hour prior to sacrifice each mouse was injected intraperitoneally with 50mg/kg BrdU (5-Bromo-2'-deoxy-uridine, Boehringer Mannheim, GmbH, Mannheim, Germany).

Long term analysis of multiple intratumoural injections in HepG2 tumours.

Mice were injected s.c. in the left flank with $1*10^7$ HepG2 cells suspended in 200µl serum-free Hanks. Every other day following tumour cell injection the mice were checked for tumour growth. Once a tumour became detectable by eye, registration of tumour growth started as described in the previous section. Calculation of tumour volume was done using the following formula: [smallest diameter]^2*[largest diameter]/2. Height was evaluated separately. Each tumour was allowed to grow upto a minimum volume of 250mm^3 and a minimal height of 3 mm. When a tumour met *both* these criteria, the mouse entered the experiment and was randomly designated to one of the three treatment groups (AdMLP.Apoptin, AdCMV.LacZ or virus dilution buffer). The same day, the first virus injection was given intratumourally, followed by four intratumoural injections on alternating days. The injections were given on alternating days to allow for resorption of the injected volume prior to the next injection. For each injection $3*10^9$ pfu in 50 µl virus in virus dilution buffer or virus dilution buffer alone was injected into the tumour tissue that looked most viable (as

judged by colour). The tumour was measured every other day until the end-criteria were reached (>1800 mm^3 and/or > 8 mm in height). At that time the mouse was sacrificed, blood was collected and the tumour was excised, weighed and fixed for further examination.

Histopathological analysis.

Paraffin-embedded sections were prepared by routine methods and stained with haematoxylin and eosin. Additionally, sections (5μm) were stained for Apoptin and ß-galactosidase expression by a three-step immunoperoxidase staining using the αVP3C antibody (produced by EurogenTec, Seraing, Belgium), and an anti-ß-galactosidase antibody (DAKO, Glostrup, Denmark). Briefly, slides were deparaffinated, washed and endogenous peroxidase activity was blocked for 20' with methanol/H_2O_2 0,3%. After rehydration, antigen retrieval was performed by a 10' incubation in boiling citrate buffer (0,01 M citric acid/0,01 M sodiumcitrate, pH=6.0). After cooling, slides were washed in PBS and incubated overnight with the antibodies anti-VP3C and anti-ß-Galactosidase in PBS/BSA1% (1:2000 for both). After washing the slides were incubated with biotinylated anti-rabbit Ig (1:400, 30'). Next, after washing, the slides were incubated with biotinylated HRP/streptavidine complex (DAKO, Glostrup, Denmark), washed with PBS and developed in di-amino carbazole (DAB). Staining of Apoptin and ß-galactosidase was scored semiquantitatively by two independent observers and expressed as percentage of positive tumour cells within a tumour. DNA-incorporated BrdU was detected by a three-step immunoperoxidase staining with the anti-BrdU monoclonal antibody IU_4 (Hycult bv, Uden, The Netherlands).[27]

Statistics.

Log rank tests were used for survival analysis. One AdMLP.Apoptin-treated mouse died 67 days after the start of treatment for no apparent reason. Since this mouse had been tumour-free for over a month, it was censored in the analysis. Based on normal probability plots one outlier (in the LacZ group) was excluded from the log rank test. All animals (including outlier) are shown in the Kaplan Meier plot (fig 5). Results were considered statistically significant at $P < 0.01$. In all cases, the investigator responsible for treatment and measurement was 'blinded' to the experimental status of the mouse.

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Chapter 4

The tumor-selective viral protein Apoptin effectively kills human biliary tract cancer cells

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Abstract

Biliary tract cancer, or cholangiocarcinoma, has a poor prognosis. Resection is the only curative treatment available at the moment, but only a minority of patients is eligible. Chemotherapy and γ-irradiation are merely palliative, as they are unable to remove the malignancy completely. Therefore a more potent therapy is warranted. The chicken anemia virus-derived protein Apoptin induces apoptosis in a wide range of human tumor cells. It is not hindered by mutations inactivating p53 nor by overexpression of Bcl-2, lesions both known to frustrate chemo- and radiation therapy. Thus, we were interested whether Apoptin could be effective against cholangiocarcinoma. After overexpression, Apoptin caused extensive cell death in three independent cholangiocarcinoma cell lines, CC-LP, CC-SW and Mz-ChA-1, regardless of their oncogenic mutations, which included inactivated p16 and p53. Moreover, co-expression of the caspase inhibitor p35 with Apoptin delayed the induced cell death, but changes in nuclear morphology still occurred early after transfection and nuclei eventually disintegrated, suggesting that Apoptin-induced cell death is not blocked by mutations in either the initiation or execution phase of apoptosis. Furthermore, we show that delivery by an adenoviral vector is an efficient method to express Apoptin in cholangiocarcinoma cells and to eradicate them. The fact that all healthy cell types tested to date are refractory to Apoptin, combined with its efficient induction of cell death in cholangiocarcinoma cell lines, makes Apoptin an attractive candidate for molecular therapy of biliary tract cancer.

Introduction

Malignancies of the bile duct give rise to high morbidity and mortality. These cholangiocarcinomas can be cured by surgery, but only in a minority of cases. Resection is particularly difficult due to dissemination of the tumor into different bile ducts [1]. At the time of diagnosis, only half of the patients are resectable, but even then the 5-year survival rate is only

around 25% [2-4]. Palliating the effects of biliary obstruction by endoscopic stenting is therefore often the only therapeutic possibility [5], especially since chemotherapy and γ-irradiation are not effective against cholangiocarcinoma [3,6,7], probably due to molecular alterations of these cells [8-10]. During carcinogenesis cells acquire traits that enable them to continue proliferation. Among others, this comprises disabling fail-safe mechanisms that normal cells have against uncontrolled growth, one of which is the induction of apoptosis [11]. Paradoxically, it is precisely this suicide program that cancer therapies such as chemotherapy and radiation employ to induce cell death. Thus, when tumor cells have obtained anti-apoptotic lesions they usually become resistant to therapy [8,12,13].

Therefore, successful treatment of cholangiocarcinoma should be based on an agent that is not hindered by these features. In addition, it is important that this agent shows selectivity in killing tumor cells and not healthy cells. Cholangiocarcinoma are surrounded by highly proliferating cholangiocytes that will be sensitive to treatments targeting cell division. In addition, cholangiocarcinomas are located near to or within the liver, which is often particularly sensitive to cytotoxic therapies because of its detoxifying function. The sensitivity of these adjacent tissues to the anticancer agent limits the therapeutic dose that can be administered and thus should be as low as possible [6,14].

Currently, several innovative strategies are being examined for treatment of cholangiocarcinoma. One such approach uses the combination of radiation therapy with novel radiosensitizers such as topoisomerase inhibitors, taxanes and nucleoside analogs [6]. In addition, other approaches have been designed to target specific tumor properties by inhibiting growth factor receptors, cyclooxygenases and angiogenesis [15-18]. Moreover, therapies based on gene delivery use prodrug-enzyme combinations like 5-fluorouracil and cytosine deaminase or the expression of specific membrane receptors to enhance tumor uptake of radiolabeled peptides [19,20]. Although many approaches show encouraging results in cell culture and preclinical studies, no clinical benefits have yet been reported.

In this study, we investigated whether the viral protein Apoptin could be a potential anti-tumor agent for cholangiocarcinoma. Apoptin, one of three proteins encoded by the chicken anemia virus (CAV), efficiently kills a wide range of human tumor cells, including osteosarcoma, breast carcinoma, lymphoma and hepatoma [21]. It was shown that cell death induced by Apoptin displays the characteristics of apoptosis. Namely, nuclei condense and eventually show typical DNA laddering and the proteases involved in execution of the suicide program, called caspases, are activated [22]. Interestingly, there are no known mutations in tumor cells that inhibit Apoptin-induced apoptosis. For instance, non-functional p53 or overexpression of Bcl-2, characteristics known to inhibit apoptosis induced by chemotherapy, do not inactivate Apoptin [23-26]. Given that chemotherapy and radiation are not effective in treating cholangiocarcinoma, Apoptin could be an attractive alternative.

In addition to its potent pro-apoptotic properties, Apoptin exhibits remarkable tumor-specificity. When Apoptin is over-expressed in non-transformed, primary cells, no adverse effects can be detected [27]. Moreover, primary fibroblasts stably transfected with Apoptin continue to

proliferate up to the same number of passages as their control counterparts (B. Klein and M. Noteborn, unpublished data). In addition, Apoptin was nontoxic in human keratinocytes, thymocytes, smooth muscle cells, endothelial cells, mesenchymal stem cells and hepatocytes when tested *in vitro* [27,28]. This tumor-selectivity renders Apoptin suitable for non-targeted or systemic therapy. As a first step to determine whether Apoptin might be useful against cholangiocarcinoma, we analyzed whether biliary tract cancer cells are indeed sensitive to Apoptin. In this study we show that Apoptin induces robust apoptosis in several cholangiocarcinoma cell lines *in vitro*, and moreover, that replication-deficient adenoviruses expressing Apoptin constitute an effective means to kill these cells.

Results

Apoptin induces cell death in cholangiocarcinoma cell lines

To determine whether cholangiocarcinoma-derived cell lines are sensitive to apoptosis induction by Apoptin, we analyzed three cell lines CC-SW [29], CC-LP [29] and Mz-ChA-1 [30]. CC-SW and CC-LP both are moderately differentiated adenocarcinoma originating from bile duct epithelium. CC-SW was derived from a patient without treatment prior to resection and CC-LP was isolated from a patient who underwent chemotherapy with 5-fluorouracil and leucovorin prior to surgery. Mz-ChA-1 was cultured from a gall bladder adenocarcinoma metastasis. Several oncogenic mutations have been described for these cell lines, including hypermethylation of the p16 promoter in CC-LP [38] and the disruption of the transforming growth factor beta (TGF-β) signaling pathway in Mz-Cha-1 [39]. In addition, we observed overexpression of p53, indicative of non-functional p53 protein, in CC-SW and Mz-Cha-1, and a complete absence of p53 protein in CC-LP cells (data not shown). In general, p53 mutations are frequently found in cholangiocarcinoma [38,40-42], as well as overexpression of Bcl-x_L [43] and HER-2/neu [17].

The three cholangiocarcinoma cell lines were transfected with plasmids encoding Apoptin or desmin, the structural protein of muscle cells, as a non-apoptosis inducing negative control. On several days after transfection cells were fixed and analyzed by indirect immunofluorescence using specific antibodies for either Apoptin or desmin. Positive cells were judged for nuclear morphology based on DNA staining with DAPI, which stains intact DNA strongly, apoptotic DNA weakly and allows detection of DNA fragmentation (figure 1a). Two days after transfection, the percentage of apoptotic nuclei among Apoptin positive cells was already considerable higher compared to desmin-positive cells (figure 1b). Several days later, Apoptin induced cell death up to 80-90% in all three lines. These data demonstrate that regardless of the molecular alterations that characterize these tumor cell lines, they are nevertheless sensitive to induction of apoptosis by Apoptin.

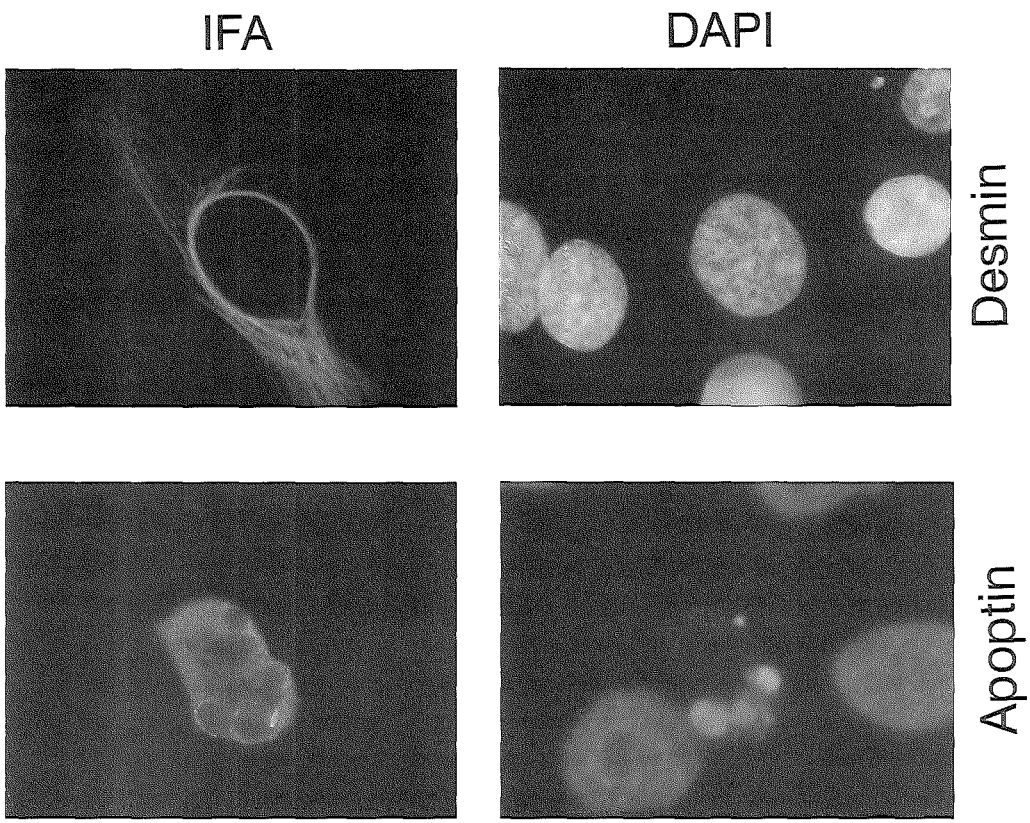

Figure 1A. Apoptin induces cell death in three cholangiocarcinoma cell lines. Plasmids encoding Apoptin and desmin were transfected into CC-LP, CC-SW and Mz-ChA-1 cells. Expression was visualized by indirect immunofluorescence. Representative images are shown from CC-SW cells expressing Apoptin and desmin, but results were similar in CC-LP and Mz-ChA-1 cells. Apoptotic morphology was determined by DAPI staining.

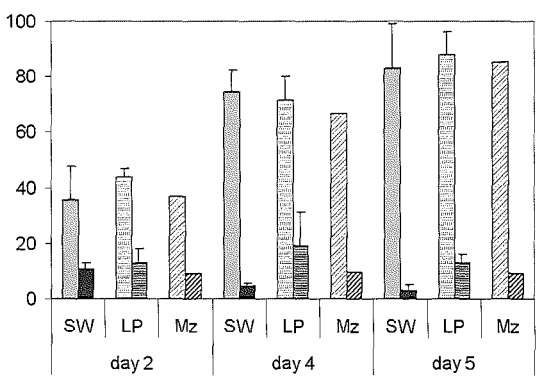

Figure 1B Apoptin induces cell death in three cholangiocarcinoma cell lines. Percentage of apoptotic nuclei in Apoptin- (grey bars) or desmin- (black bars) transfected cholangiocarcinoma cell lines (CC-SW: filled, CC-LP: horizontal stripes and Mz-ChA-1: diagonal stripes) measured on several days after transfection. In each experiment at least 100 cells expressing Apoptin or desmin were examined and the values are the mean of three independent experiments for CC-SW and CC-LP and of two independent experiments for Mz-ChA-1.

Apoptin-induced cell death is delayed but not abrogated by inhibition of caspases

Since conventional chemo- and radiation therapy are thwarted by mutations in the apoptotic pathway, we investigated whether a block downstream in the apoptosis pathway would inhibit the anti-tumor activity of Apoptin in cholangiocarcinoma cell lines. To this end, caspase inhibitor p35, a gene derived from baculovirus whose product blocks both initiator and execution caspases [44], was co-expressed with Apoptin in CC-LP cells. Again desmin was used as negative control. Death of CC-LP cells induced by Apoptin was significantly delayed when p35 was co-expressed (figure 2a). This indicates that cell death caused by Apoptin in cholangiocarcinoma occurs at least in part *via* the activation of caspases, and thus uses components of the apoptotic machinery of the cell as it does in osteosarcoma [22]. However, coexpression of p35 with Apoptin did not lead to a decrease in cells with abnormal nuclei compared to expression of Apoptin alone. In fact, although typical apoptotic morphology was absent, these cells displayed slight changes in nuclear morphology that were not observed in cells coexpressing p35 and desmin (figure 2b). Furthermore, four days after transfection, the percentage of dead cells expressing Apoptin increased substantially above background. Thus, under these conditions, caspase inhibition seems to delay Apoptin-induced death rather than blocking it completely.

Anti-tumor effect of Apoptin expressed by an adenoviral vector

Because Apoptin is not endogenously present, it will have to be introduced into tumor cells. One of the most efficient delivery methods available at the moment is the adenoviral vector system. To explore the possibility whether gene therapy with Apoptin might be effective against cholangiocarcinoma, we used a replication-deficient adenovirus vector expressing Apoptin or the LacZ gene as a negative control. Three days after infection, the number of CC-LP cells dramatically decreased with increasing dose of viral vectors expressing Apoptin until no cells were left, while with the same or even higher doses of control virus, the cells remained unharmed (figure 3). Similar results were obtained in cell viability assays using the WST-1 method (data not shown). These data provide a proof of principle that when Apoptin is efficiently transduced to tumor cells, they can all be eliminated.

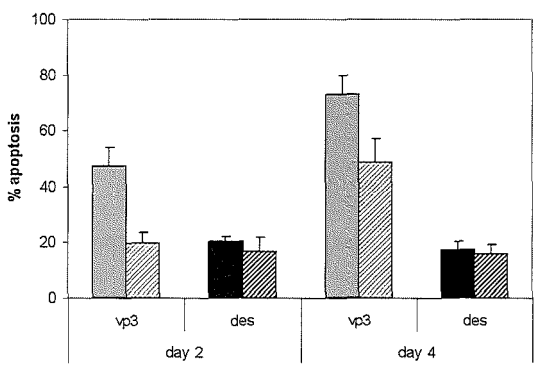

Figure 2A. Effects of the caspase inhibitor p35 on Apoptin-induced cell death. Induction of apoptosis by Apoptin (grey bars) in CC-LP cells is delayed by coexpression of p35 (striped bars). Desmin (black bars) constitutes a non-apoptosis inducing control and thus represents transfection toxicity. Values represent the mean of three independent experiments in which at least 100 positive cells were examined

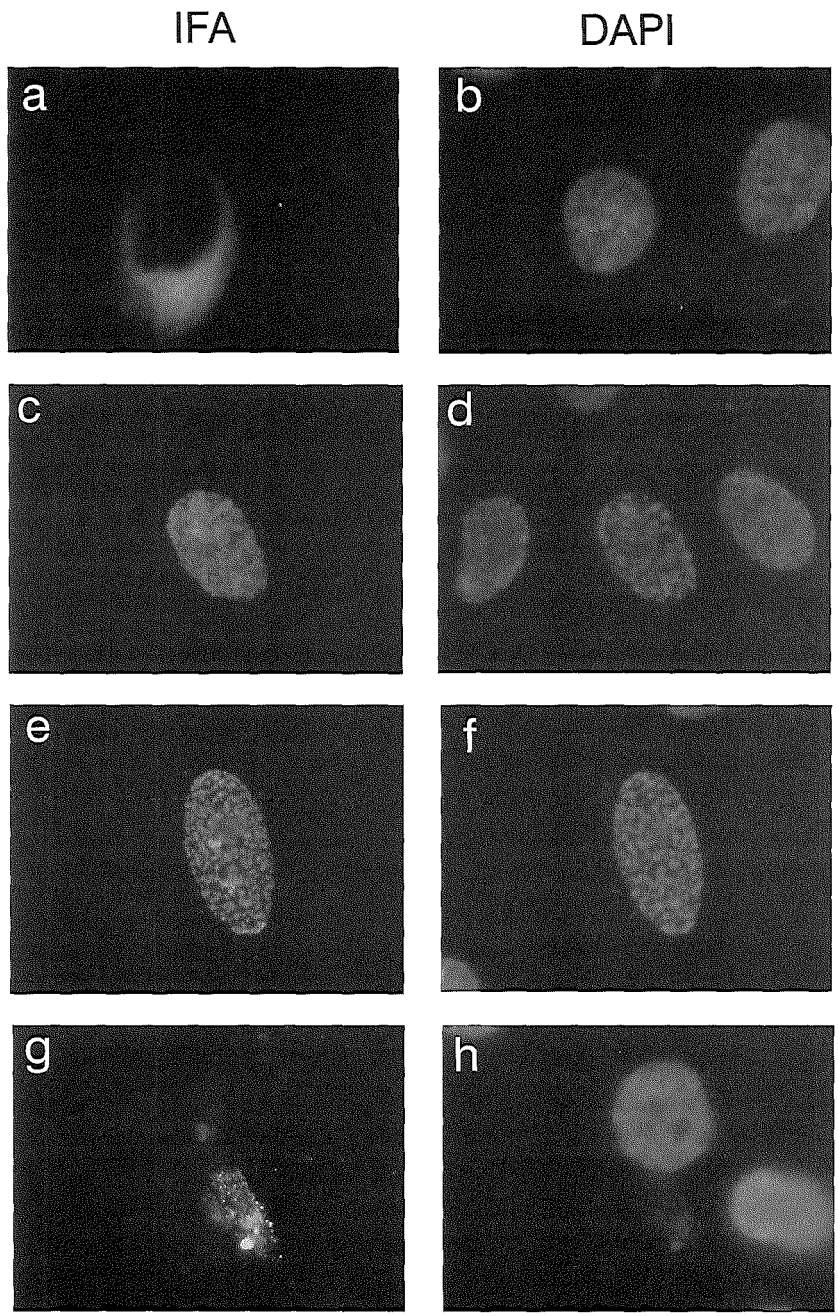

Figure 2 B Effects of the caspase inhibitor p35 on Apoptin-induced cell death (cont.) Co-expression of desmin and p35 does not lead to altered nuclear morphology (a+b). When Apoptin is co-expressed with p35, the overall number of apoptotic cells is reduced but the non-apoptotic cells show changes in nuclear morphology (c+d and e+f). Approximately half of the cells expressing Apoptin and p35 still undergo apoptosis four days after transfection (g+h).

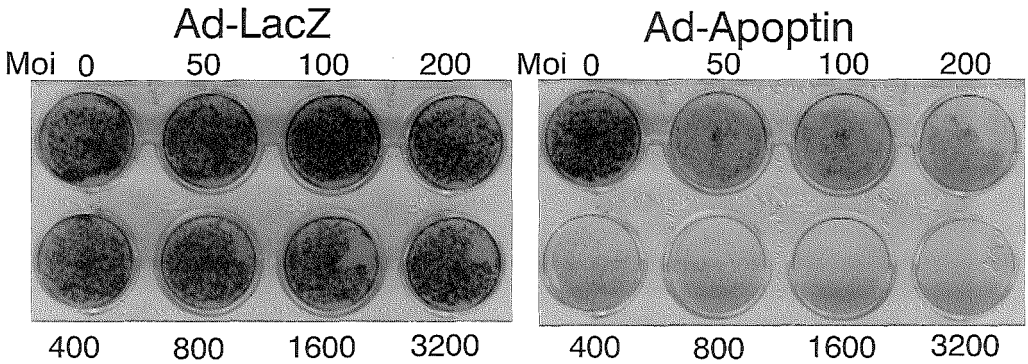

Figure 3. Expression of Apoptin by adenovirus vectors efficiently kills CC-LP cells. **CC-LP cells were infected with an increasing number of viral particles per cell (m.o.i.) from a replication-deficient adenoviral vector expressing Apoptin (Ad-Apoptin) or marker protein β-galactosidase (Ad-LacZ). Remaining cells were visualized by Giemsa staining**

Discussion

The study described here shows that Apoptin could be a candidate for treatment of cholangiocarcinoma. We first showed that cholangiocarcinoma cells can be effectively killed by Apoptin. Cholangiocarcinomas have been shown to harbour multiple anti-apoptotic mutations, including p53 mutations and upregulated Bcl-XL [38,43], lesions that can lead to drug resistance [8,12]. Importantly, the three independent cholangiocarcinoma cell lines tested here were equally sensitive to Apoptin-induced apoptosis, including CC-LP cells which failed to respond to chemotherapy *in vivo*. Even when caspases were blocked by a broad-spectrum inhibitor such as p35, the cells still died, although the process was delayed. Probably cell death is more efficient when caspases cleave crucial substrates such as PARP, Bid and DNase, subsequently disabling several components of the cell simultaneously, including those involved in metabolism and DNA integrity [45]. The residual apoptosis induced by Apoptin could be explained by incomplete inhibition by p35 under these transient coexpression conditions. However, the effect of p35 is detectable by the nuclear changes that occur in approximately all non-apoptotic Apoptin-expressing cells, which are absent when p35 is not co-expressed. Eventually, the nuclei disintegrate completely in a considerable percentage of these cells with altered nuclear morphology, suggesting Apoptin causes such damage that the cells are no longer viable. Significantly, the delay of cell death rather than inhibition of Apoptin-induced apoptosis by p35 has been observed previously in osteosarcoma cells [22], and several groups have reported that caspase inhibition after an apoptotic insult leads to loss of clonogenicity [46,47]. Thus, also in cholangiocarcinoma, mutations in both the decision and execution phase of apoptosis are not likely to confer resistance to Apoptin.

The therapeutic window of an anticancer agent is not only determined by its potency, but also by its toxicity to healthy surrounding tissues. Notably, it was demonstrated that human hepatocytes are refractory to Apoptin when injected as a recombinant protein *in vitro* [28]. In addition, no

hepatotoxicity was observed after intravenous administration of an adenoviral vector expressing Apoptin in rats [35]. In the future, the sensitivity of cholangiocytes to Apoptin will need to be assessed. Due to technical difficulties with obtaining sufficient cholangiocytes and their limited culture time, this question can be best addressed *in vivo*. In order to achieve this, an efficient delivery method is required. As described below, several approaches are being pursued at the moment. If Apoptin indeed shows the same specificity for cholangiocarcinoma compared to cholangiocytes as has been demonstrated, for instance, in tumorigenic fibroblasts and keratinocytes compared to their normal counterparts [27], Apoptin may be applicable in diagnosis of cholangiocarcinoma as well. At the moment diagnosis is frequently based on suspicion because endoscopic brush cytology and/or biopsies and imaging studies are often negative for malignancy [48,49].

The tumor-selectivity of Apoptin renders it suitable for systemic therapy, which in the case of non-resectable cholangiocarcinoma would be preferable. Alternatively, semi-regional treatment by occlusion of the bile ducts would also be a possibility. In any case, the major prerequisite at the moment is a suitable vector to deliver the *Apoptin* gene. In this study, we show that adenoviral transduction of Apoptin is an effective method of introducing Apoptin into cholangiocarcinoma cells *in vitro*, a procedure that subsequently causes the elimination of all tumor cells. Others have also reported that cholangiocarcinoma cells can be efficiently transduced by adenovirus [19,50], indicating that the receptor is present. We have shown that replication-deficient adenovirus vectors expressing Apoptin have significant anti-tumor effects against xenografted hepatoma, in some cases leading to complete regression [36]. However, these effects were depended on efficient penetration and transduction, which was only achieved in a fraction of the tumors. Therefore, it is not likely that replication-deficient adenoviral vectors will be very effective in delivering Apoptin to all cells within a cholangiocarcinoma, especially with its difficult topology. A promising strategy would be to use conditionally replicating adenoviruses (CRADs)[51] that would nevertheless require retrograde injections into the cholangiocarcinoma tumor mass, but would then be able to spread to deeper layers and more distal bile ducts. Inclusion of Apoptin into these vectors could in theory add essential potency and specificity to these vectors. Previous studies have already shown that the production of adenoviral particles is completed prior to the induction of cell death by Apoptin [35], rendering this a technical feasible approach. Alternatively, fusion of Apoptin to a peptide that confers the ability to cross cell membranes to the protein would eliminate the need for intra-tumoral injections. Efficient transduction by proteins fused to the TAT-peptide of virtually all tissues in a living mouse has been described [52], and the selectivity of Apoptin renders it suitable to such a systemic approach.

In conclusion, the lack of efficient treatment for cholangiocarcinoma warrants the evaluation of alternative treatments. We propose that Apoptin could be a potential candidate, as it is highly effective in killing several cholangiocarcinoma cell lines, in spite of their molecular alterations. The selectivity of Apoptin observed so far implies a large therapeutic window, although this needs further experimentation for cholangiocarcinoma. The development of an efficient delivery method for Apoptin could potentially provide a new treatment modality for this incurable tumor.

Experimental procedures

Cells and cell culture

The cholangiocarcinoma cell lines CC-LP [29], CC-SW [29] and Mz-ChA-1 [30] and adenovirus producer cell lines PER.C6 [31] and 911 [32] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Rockville, Md) in a 5% CO2 atmosphere at 37°C. For immunofluorescence, cells were grown on glass microscope slides.

Plasmids and transfection

Expression plasmid pCMV-vp3 contains CAV DNA sequences encoding Apoptin (nt 427-868) under control of the cytomegalovirus (CMV) enhancer/promoter [26]. pCMV-desmin encodes desmin, a component of type II intermediate filaments, and was used as a negative control for the induction of apoptosis [33], pCMV-neo is the empty vector control [26]. The subcloning of the cDNA of p35 in pCMV has been described previously [22]. pAdApt-vp3 was generated by ligation of the Apoptin-*Bam*H1 fragment from pCMV-vp3 into the adenoviral transfer vector pAdApt constructed by Introgene (now Crucell Holland BV).

Plasmid DNA was purified by centrifugation in a CsCl gradient and transfection was carried out by complexing with FuGENE 6 Transfection Reagent according to the manufacturer's protocol (Boehringer Mannheim, Almere, The Netherlands). In co-transfections, pCMV-p35 or pCMV-neo was used in three-fold excess to the plasmid encoding the protein that was stained by immune fluorescence (Apoptin or desmin). The DNA:Fugene ratio was 1:3 in all cases.

Indirect immunofluorescence and DAPI staining

Indirect immunofluorescence was performed as described previously [34]. To demonstrate the presence of Apoptin and establish its cellular localization in transfected cells, the cells were fixed with 80% acetone. The indirect immunofluorescence assay (IFA) was performed with hybridoma culture supernatant containing the mouse monoclonal antibody (mAb) 111.3 for Apoptin and with a 100-fold dilution of mAb 33 (Monosan, Uden, The Netherlands) for desmin. Fluorescein-isothiocyanate-labeled goat anti-mouse antibody (Jackson Immunoresearch Laboratories Inc., West Grove PA, USA) was used as second antibody. Nuclear DNA was stained with 1 μg/ml 2,4-diamino-2-phenylindole (DAPI) in 2% 1, 4 diazabicyclo-[2,2,2]-octane in glycerol/0.1 M TrisHCl pH 8.0.

Adenovirus production and infection

A replication-deficient adenoviral vector lacking the E1 region and expressing Apoptin under the regulation of the CMV promoter was generated by cotransfecting near-confluent monolayers of PER.C6 cells with the *Af*lII-ITR fragment of adenovirus type 5 and pAdApt-vp3. When cytopathogenic effects occurred, cells were harvested and lysed by freeze-thawing and the subsequent viral supernatant was serially diluted on PER.C6 cells. Individual clones were tested for Apoptin expression by immunofluorescence and for the absence of replication-competent adenovirus by PCR as described [35]. The recombinant adenoviral vector AdCMVlacZ carries the *E. coli LacZ* gene expressing β-galactosidase under control of the CMV promoter [36]. Large-scale production of adenovirus was performed according to Fallaux *et al.* [32]. Briefly, PER.C6 helper cells were infected with approximately 5 plaque-forming units per cell, in a small volume of DMEM containing 2% horse serum (HS) (heat-inactivated at 56°C for 30 min). After a 2-hour incubation, DMEM/10%FCS was added to each flask. Forty-eight hours later, the detaching cells were harvested and collected in PBS/2% HS. Viruses were isolated from the producer cells by freeze/thawing, purified by cesium-chloride gradient centrifugation and dialysis against a sucrose-containing buffer. Viral titers were determined by plaque assays essentially as described by Graham and Prevec [37]. Briefly, adenovirus stocks were serially diluted in DMEM/2% HS and added to near-confluent 911 cells in six-well plates. After 2 hours of incubation at 37°C/5% CO2, the medium was replaced by agar-containing culture medium. Plaques were scored on day 5-7 after infection.

Cholangiocarcinoma cells were counted and plated four to six hours prior to infection. At the time of infection, the appropriate number of infectious viral particles per cell (multiplicity of infection, m.o.i.) was added in a small volume of DMEM/2%HS. After a 2-hour incubation, the volume was increased to regular levels with DMEM/10%FCS. This virus-containing medium was replaced the following morning with DMEM/10%FCS.

Giemsa staining

For detection of the number of attached cells, CC-LP cells were washed twice with PBS three days after infection and air-dried. Cells were subsequently fixed in methanol/acetic acid (3:1) for 15 minutes at room temperature. Cells were

incubated with Giemsa solution (3% Giemsa (Merck, Darmstad, Germany) for 30 minutes in 1 mM Na_2HPO_4, pH 7.0). After staining, the cells were washed four times with deionized water and allowed to air-dry.

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Chapter 5

Continuous Apoptin expression in transgenic mice does not interfere with lymphocyte development and proliferation

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Abstract

Apoptin is one of few cytocidal proteins endowed with tumor-specificity. This protein is activated and induces apoptosis in a wide variety of tumor cells but leaves non-transformed cells unharmed. Currently, systemic therapies are hindered by toxicity to rapidly dividing tissues, including the immune system. Apoptin is therefore an attractive candidate for systemic cancer therapy. To investigate whether Apoptin remains inert in normal lymphocytes, even during development, activation and proliferation of these cells, we generated transgenic mice expressing Apoptin under the regulation of the H2-K^b promoter.

The Apoptin-transgenic mice were born with the expected Mendelian distribution and exhibited normal development. Robust Apoptin RNA expression was detected in thymus and spleen. Apoptin protein, however, could be detected only after concentration by immunoprecipitation. In addition, we show that the amount of Apoptin protein detected in transgenic splenocytes was significantly increased by inhibition of the proteasome. This instability of Apoptin in normal cells could be part of the explanation for its lack of activity in normal cells.

Importantly, there was no difference in B, helper T- or cytotoxic T cell numbers derived from transgenic spleens compared to spleens from wild-type littermates, suggesting that none of these cell types is sensitive to Apoptin expression during any of their developmental stages. Moreover, stimulation of B cells with LPS or stimulation of T cells with IL-2 and conA did not result in a growth disadvantage for the transgenic lymphocytes, nor did it lead to increased cell death. These data show that normal lymphocytes tolerate the expression of Apoptin during both activation and proliferation, eliminating a first hurdle for the development of systemic therapy with Apoptin.

Introduction

Chickens infected with the chicken anaemia virus (CAV) not only suffer from anaemia, but also from a depletion of thymocytes [1,2]. Thymuses isolated from CAV-infected chickens showed DNA-laddering typical of apoptosis [3]. This was also observed after expression of the viral genome in transformed chicken T cells, and when one of the three proteins encoded by CAV was expressed separately [4]. This protein was therefore named Apoptin. Subsequent studies revealed that Apoptin induced apoptosis in cells from human and rodent origin as well [5]. Strikingly, the ability to induce apoptosis is restricted to tumor and transformed cells. In these cells, Apoptin localizes to the nucleus and causes cell death. Expression of Apoptin in 'normal' diploid cells on the other hand shows a sequestration of Apoptin in the cytoplasm, sometimes appearing in vesicular structures, and the cells remain viable without any signs of apoptosis [6].

At this moment it is not clear what element of the 'transformed nature' of a cell is responsible for the activation of Apoptin that renders it capable of inducing apoptosis. However, already the selective tumor-cell killing of Apoptin offers an excellent opportunity for the development of new therapies for cancer. Current cancer therapies are, among other things, limited by their toxicity to normal tissues [7]. Innovative strategies designed to circumvent this problem aim at tumor-specific delivery of a toxic substance, or alternatively, at systemic delivery of a tumor-specific substance [8]. Apoptin is one of few proteins that may be suited for the latter approach.

In previous studies, we have shown that transfer of the *Apoptin* gene by replication-deficient adenoviruses to subcutaneous human hepatoma grafted on nude mice resulted in considerable demise of tumor cells [9]. Moreover, the Apoptin treatment conferred a significant survival benefit to the mice [10]. In addition to the absence of adverse effects of the treatment for the mice, primary rat hepatocytes remained unscathed by adenovirally expressed Apoptin, both *in vitro* and *in vivo*. These data confirm that Apoptin is capable of eradicating tumor cells *in vivo*. Nevertheless, the studies with the replication-deficient adenovirus expressing Apoptin emphasized the main curb of gene therapy at this moment, namely the efficiency of transduction. Virus spread through solid tumors is severely limited, so only a small percentage of the entire tumor mass can be infected. Thus, new treatment modalities need to be developed that more efficiently transduce Apoptin to all tumor cells. Based on the unique properties of Apoptin, these strategies will be focussed on systemic delivery, such as insertion of the *Apoptin* gene into replicating vectors, or modification of Apoptin protein so it can pass cell membranes.

One of the normal tissues affected by systemic anticancer therapies is the immune system [7]. Given that CAV causes apoptosis in chicken thymocytes, we wanted to know if problems can be expected in the lymphoid department of the body when Apoptin is employed for systemic therapy. To include all stages of differentiation and proliferation of lymphocytes, a transgenic approach was taken to answer this question.

We found that transgenic mice expressing *Apoptin* under the regulation of the H2-K^b promoter are viable, express Apoptin mainly in the lymphoid organs, and have normal B and T cell populations. In addition, the biochemical properties of Apoptin in these transgenic mice provide a possible explanation for the tumor-specificity of Apoptin, namely its short half-life in

normal cells. The observation that even during stimulation of B and T cells Apoptin does not become active offers great promise for systemic therapy with Apoptin.

Results

Apoptin-transgenic mice are viable

To obtain insight in the effects of prolonged expression of Apoptin during differentiation and proliferation of lymphoid cells, Apoptin-transgenic mice were generated. Because we were not initially interested in the effects of Apoptin on the embryonic development a promoter was chosen that starts protein expression late in embryogenesis. This promoter is the H-2K^b promoter, the murine homologue of human MHC class I. Transgenic mice have been generated previously using the same construct [11]. Expression of the transgene was hardly detectable during the first 11 days of embryogenesis, after which it rapidly increased. Due to a partial promoter region and positional effects at the site of integration [12], expression does not occur in all tissues as would be expected from a general gene product like MHC class I. Rather, expression in adult mice was high in spleen, lymph nodes, lung and liver which makes this construct suitable to test our hypothesis on the effects of Apoptin in lymphocytes.

The *Apoptin* gene was inserted in the first exon of the H-2K^b promoter. To test whether Apoptin expressed by this construct is capable of inducing apoptosis, we transiently transfected it into cultured Saos-2 osteosarcoma cells. Apoptin expressed by the H-2K^b promoter induced apoptosis to a similar extent as when expressed from a CMV promoter (fig. 1a), demonstrating that Apoptin is functional.

After pronuclear injection seven transgenic founders were born among 51 pups. H-2K^b-Apoptin mice are born in the expected Mendelian ratios, are fertile, and do not show any gross morphological or behavioural abnormalities (data not shown). The presence of the *Apoptin* gene was confirmed by Southern blotting (fig. 1b) and PCR analysis (fig. 1c). Sequence analysis revealed an intact *Apoptin* gene in different founders (data not shown).

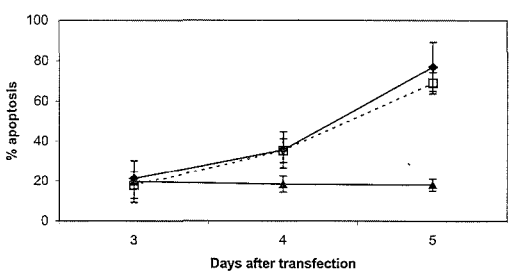

Figure 1A Induction of apoptosis by pH2-K^b-Apoptin.

Osteosarcoma (Saos-2) cells were transiently transfected with pH2-K^b-Apoptin (filled diamond), pCMV-Apoptin (open square) or pCMV-desmin (filled triangle). Cells positive for the transfected protein as judged by immunofluorescence were scored for apoptosis based on nuclear morphology on day 3, 4 and 5 after transfection.

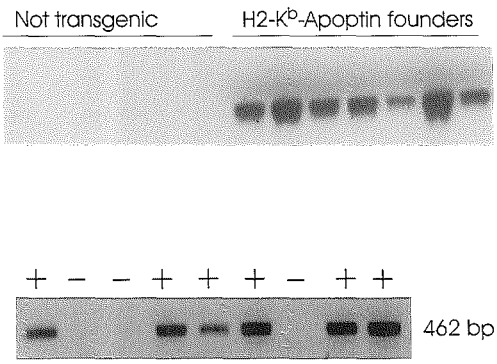

Figure 1B Characterization of H2- K^b-Apoptin mice by Southern and PCR analysis.

(B) H2-K^b-Apoptin transgenic mice were identified by hybridising XbaI-digested DNA with a ^{32}P-labeled Apoptin probe on Southern blot.

(C) Additionally, a PCR method was developed that also specifically detects H2-K^b-Apoptin DNA. (+) indicates H2-K^b-Apoptin founders and (-) wild-type littermates.

Apoptin is primarily expressed in lymphoid tissues

To determine Apoptin expression in different tissues, cytoplasmic RNA was isolated from different organs and hybridised with a ^{32}P-labeled Apoptin probe. Apoptin mRNA could easily be detected in thymus, spleen and lung (fig. 2a). A more detailed insight in what cell types express Apoptin was obtained by RNA in situ hybridization (fig. 2b). In spleen sections, double staining with an antibody against B cells demonstrated that virtually all B cells express Apoptin. T cells were stained with an antibody directed against CD3 in both spleen and thymus sections. Double-staining with RNA in situ hybridization revealed that a subset of T cells express Apoptin (fig. 2b).

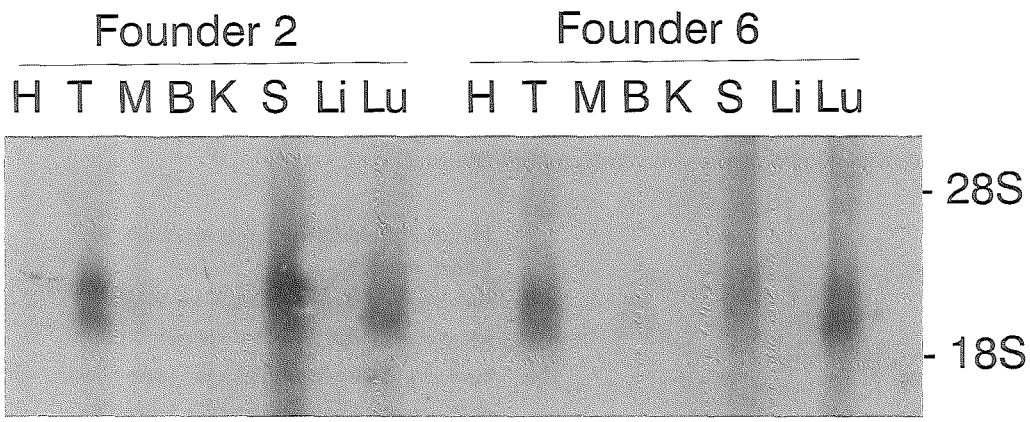

Figure 2A. RNA expression profile of H2- K^b-Apoptin mice.

Total RNA was isolated from heart (H), thymus (T), muscle (M), brain (B), kidney (K), spleen (S), liver (Li) and lung (Lu) from two H2-K^b-Apoptin mice and analysed by Northern blot using a ^{32}P-labeled Apoptin probe.

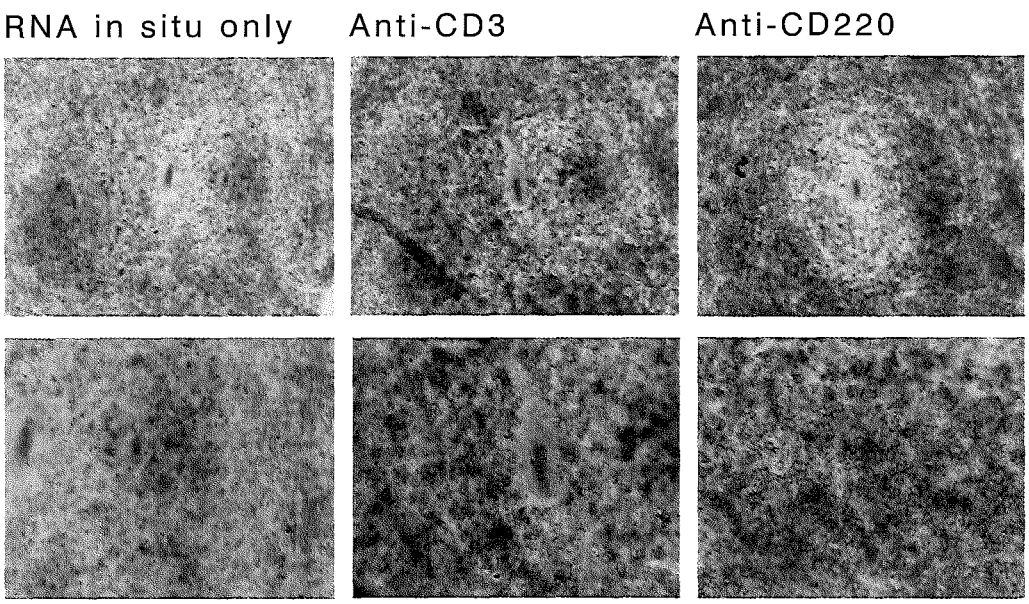

Figure 2B. RNA in situ expression in the spleen of H2-K^b-Apoptin mice.

Sequential sections from the spleen of a H2-K^b-Apoptin mouse were hybridised with a dig-labelled RNA antisense probe to detect Apoptin mRNA (purple signal) and were co-stained with antibodies against CD3 and CD220 to identify T and B cells, respectively (brown signal). The upper panel shows images taken at a 20x magnification; the images of the lower panel are taken from the same sections but magnified 40x. RNA *in situ* hybridisations of spleen sections from wild-type mice with the anti-sense probe were negative, as were hybridisations of sections from H2-K^b-Apoptin mice with the sense probe (data not shown).

Apoptin is unstable in transgenic tissues

Although the Northern blot results (fig. 2a) show a robust expression of Apoptin in these mice, Apoptin protein could not be detected by immunohistochemistry on sections of transgenic tissues. This is not likely due to unavailability of the epitope, because in previous experiments Apoptin protein was detectable in tissue sections after adenovirus-transduction (ref.[10] and S. Rutjes, unpublished data), demonstrating that the antibody should be able to recognize Apoptin under these conditions. However, concentrating the amount of Apoptin protein by immunoprecipitation from freshly isolated spleen, lung or cultured splenocytes yielded a weak signal on Western blot, albeit with low reproducibility (fig. 3a). Since the combination of high RNA expression with hardly detectable protein expression points in the direction of an unstable protein, we tested if Apoptin could be stabilized to a detectable level in the transgenic lymphocytes by inhibition of protein degradation. Indeed, after inhibiting the proteasome with CBZ/LLL or lactacystin, a significant amount of Apoptin protein was detectable (fig. 3b), indicating that although the protein is expressed from the RNA it is unstable in these cells.

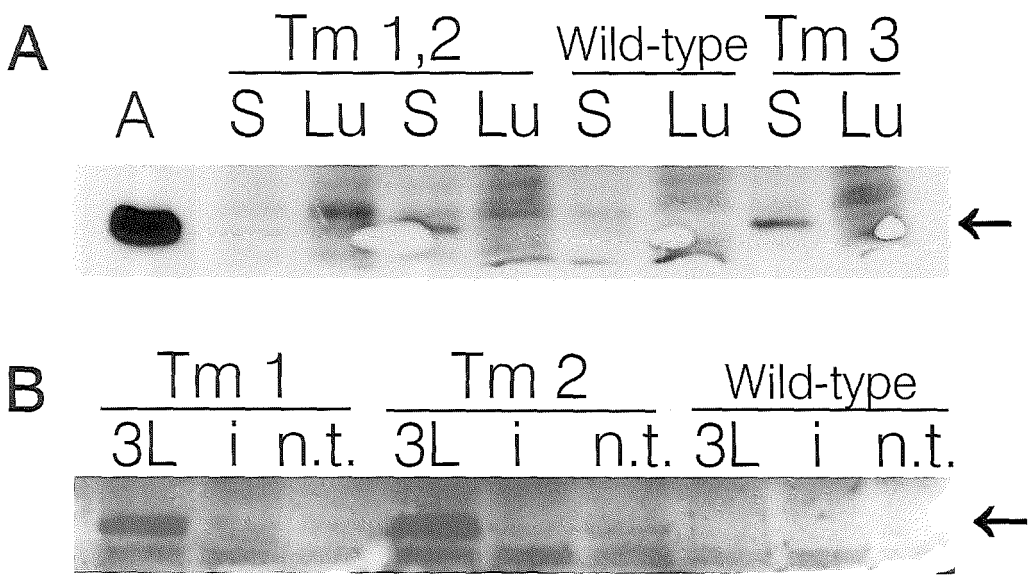

Figure 3. Apoptin protein expression in H2-K^b-Apoptin mice.

(A) Freshly isolated splenocytes (S) or lung frozen in liquid nitrogen and pulverized (Lu) from three H2-K^b-Apoptin mice (Tm 1, 2 and 3) and one wild-type mouse were lysed and subjected to immunoprecipitation using an Apoptin-specific rabbit antibody. Samples are subsequently run on Western blot and stained with mouse monoclonal 111.3 directed against Apoptin. Lysate from Saos-2 cells tranfected with pCMV-Apoptin (A) serves as a positive control for Apoptin detection on Western blot. The arrows indicate the molecular weight of Apoptin (16 kD).

(B) Splenocytes derived from two H2-K^b-Apoptin mice (Tm 1 and 2) and from 1 wild-type mouse were treated with proteasome inhibitor CBZ/LLL (3L), Interferon-γ (i) or not treated (n.t.) and subsequently lysed, immunoprecipitated and subjected to Western blot analysis as described in figure 3A.

A separate set of experiments shows that Apoptin can in fact be ubiquitylated, a prerequisite for recognition by the proteasome. SV40-transformed fibroblasts were transfected with Apoptin and HA-tagged ubiquitin. After 48 hours a proteasomal inhibitor was added to facilitate detection by accumulating ubiquitylated proteins. Apoptin was immunoprecipitated from cell lysates and staining of HA-ubiquitin on Western blot revealed Apoptin-ubiquitin conjugates (fig. 4). Proteins known to be ubiquitylated and degraded by the proteasome, like p53 and c-myc, also portray a high molecular weight smear after proteasomal inhibition (fig. 4 and ref. 13). It represents the molecule targeted for degradation, in this case Apoptin, with ubiquitin chains of various lengths attached to it.

Apoptin does not interfere with B and T cell development

Given that the spleen has the highest expression of Apoptin RNA and protein in H2-K^b-Apoptin transgenic mice, we investigated whether Apoptin expression caused any deleterious effects in

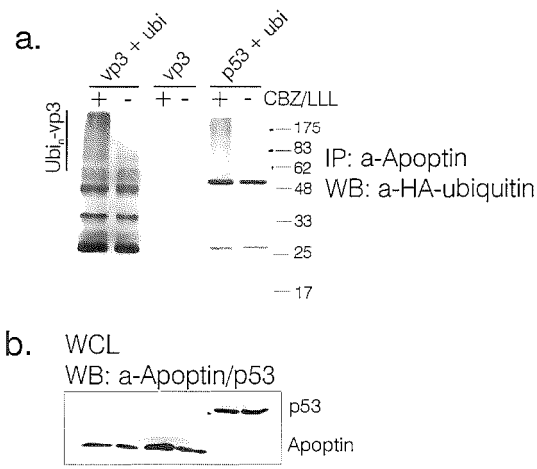

Figure 4. Apoptin is ubiquitylated and degraded by the proteasome.

SV40-transformed fibroblasts were cotransfected with Apoptin (vp3) and HA-ubiquitin (ubi), Apoptin (vp3) alone, or p53 with HA-ubiquitin (ubi), in duplo. After 48 hours proteasome inhibitor (CBZ/LLL) was added to one sample of each condition and incubated overnight. After lysis, part of the whole cell lysates (WCL) was saved as a control for transfection efficiency, the rest was subjected to immunoprecipitation (IP) with α-VP3C for samples transfected with Apoptin or DO-1 for samples transfected with p53. The western blot (WB) of the IP was stained with α-HA (a) and the blotted whole cell lysates with α-Apoptin and α-p53 (b).

splenocytes. Since the bulk of spleen cells comprise B and T cells, and the deletion of any of these cells would not lead to an immediately apparent phenotype, we first looked with FACS analysis whether all major cell types were present. As can be seen in figure 5, no differences between transgenic and wild-type littermates were found in the numbers of B cells, helper- or cytotoxic T cells, demonstrating that Apoptin does not interfere with the development of these lymphocytes.

Apoptin does not induce apoptosis in proliferating B and T cells

Next, it was determined whether stimulation of these cells might activate Apoptin to cause apoptosis. B cells were magnetically selected from wild-type and transgenic spleens and stimulated with lipopolysaccharide (LPS). The growth curves in figure 6a show that proliferation of B cells is not hindered by Apoptin expression, since the transgenic cells multiply to the same extent as wild-type control cells. Moreover, although the cells gradually die in culture, Apoptin does not cause additional cell death in these stimulated B cells (fig. 6a). The same holds true for the concanavalin A and IL-2 activated T cells from B cell-depleted spleens. The growth kinetics of the T cells in culture varied significantly between experiments; therefore, the cultures from different spleens are depicted separately. Again, the transgenic cells proliferate with similar kinetics compared to cells from the wild-type littermate (fig. 6b). Despite the difference in growth kinetics, the increase in cell death due to cell culture is remarkably consistent between the T cell cultures. Evidently, cell death was not enhanced by expression of Apoptin. Taken together, these

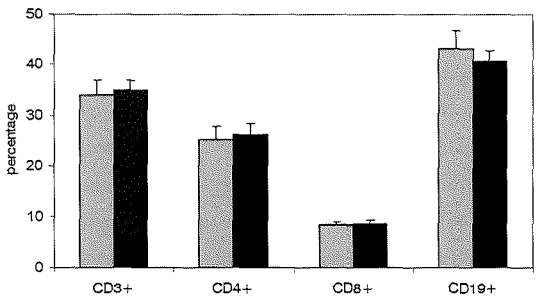

Figure 5. Presence of different cell types in spleens derived from H2-K^b-Apoptin and wild-type mice.

Splenocytes from three H2-K^b-Apoptin (grey bars) and three wild-type littermates (black bars) were stained with labelled antibodies directed against CD3 (all T cells), CD4 (helper T cells), CD8 (cytotoxic T cells) and CD19 (B cells) and analysed by FACS. The percentage of positive cells relative to the total cells counted is depicted

data show that Apoptin expression does not interfere with the differentiation of lymphocytes and, importantly, that activation of B and T cells does not trigger Apoptin to induce apoptosis.

Discussion

The generation of transgenic mice expressing Apoptin allows us to address several important questions. Up to date, all studies on Apoptin have been performed by transient expression. This restricts the data to cell types that can be subjected to *in vitro* culture, which especially for primary cells is usually of short duration. Possibly, some normal cells will be sensitive to Apoptin after a longer expression period. Specifically, tumor cells do not often acquire entirely new traits but usually show simultaneous reactivation of different pathways. These pathways are present in normal cells but are usually carefully guarded and/or inhibited except for the short period when they are necessary, for instance to divide (sustain cell number), invade (immune cells) or acquire an extended lifespan (reproductive cells) [14]. It is therefore conceivable that Apoptin might be activated during certain stages of proliferation or differentiation of normal cells in the body. For any future therapy based on Apoptin it is imperative to know if and where normal conditions exist that allow Apoptin activation, giving rise to toxic side effects of systemic treatment. This information can then be used to choose the best delivery system, thereby avoiding the cells that would be sensitive. Because the immune system is often adversely affected as a side effect of cancer therapy, we decided to study these cells first.

In this study, we show that mice expressing *Apoptin* under the regulation of the H2-K^b promoter are viable and apparently healthy. As expected, the expression is highest in lymphoid organs. Interestingly, we observed that Apoptin protein is unstable in these cells. It is not likely that this is an artefact of these transgenic mice, since no mutations in the *Apoptin* gene were found in any of the founders. Additionally, the construct used to generate the mice induced significant apoptosis in tumor cells, indicating that Apoptin expressed by this promoter is functional. Furthermore, degradation via the proteasome is a highly regulated process. The fact that Apoptin is ubiquitylated shows that there is an E3 ligase present in cells that is able to recognize the protein and conjugate ubiquitin specifically to one or more lysine residues of

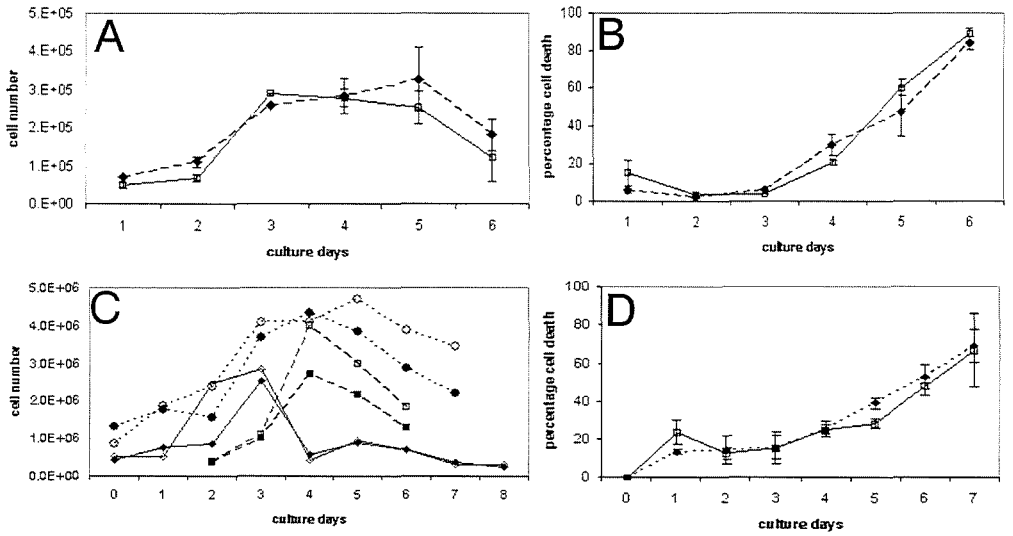

Figure 6A+B. Faith of B cells derived from H2-Kb-Apoptin and wild-type mice after in vitro stimulation with lipopolysaccharide (LPS).

A: Growth curves of B cells from H2-Kb-Apoptin (open square) and wild-type (black diamant) mice after positive selection with CD19-microbeads and culture in medium supplemented with 5 µg/ml LPS.

B: Percentage of B cells from H2-K^b-Apoptin (open square) and wild-type (black diamant) mice undergoing cell death during culture.

Figure 6C+D. Faith of T cells derived from H2-Kb-Apoptin and wild-type mice after in vitro stimulation with concanavalin A and IL-2.

C: Growth curves of T cells from H2-K^b-Apoptin (open square) and wild-type (black diamant) mice in three separate experiments. Each symbol (diamant, circle or square) represents two littermates, one wild-type (closed symbol) and one Apoptin-transgenic (open symbol). Splenocytes were depleted from B cells with Goat-anti-mouse-beads and cultured in the presence of con A and IL-2.

D: Percentage of T cells from H2-K^b-Apoptin (open square) and wild-type (black diamant) mice undergoing cell death during culture.

Apoptin. In a separate study, microinjection of recombinant Apoptin protein also revealed increased degradation of Apoptin in normal fibroblasts compared to tumor cells (Zhang et al, submitted). Taken together, these data indicate that instability of Apoptin may be a general phenomenon in normal cells.

Recent data shows that Apoptin is differentially modified, namely phosphorylated specifically in tumor cells [15]. When constitutive phosphorylation is mimicked by inserting a glutamic acid at the site of phosphorylation, this mutant Apoptin is now able to induce apoptosis in normal cells as well. Consequently, tumor-specific activation of Apoptin could be the result of a kinase that is specifically upregulated in tumor cells. Alternatively, it is also possible that the Apoptin protein is not available for the kinase in normal cells due to binding to other molecules in the cell.

Nonetheless, it should be noted that a non-phosphorylatable mutant of Apoptin can still kill tumor cells and remains inactive in normal cells, indicating that phosphorylation is not the only determinant of the tumor-specificity of Apoptin. The instability of Apoptin in normal cells may contribute to the specificity as well. It will be interesting to see whether phosphorylation can prevent Apoptin from ubiquitylation, or reversely, whether ubiquitylation of Apoptin in normal cells prevents phosphorylation. It is even conceivable that both processes bestow specificity on the protein separately, further reducing the risk of Apoptin activation in normal cells at any stage of their development.

As stated previously, the tumor-specificity of Apoptin makes it exquisitely suitable for systemic therapy. To determine whether continued expression of Apoptin in lymphocytes poses a problem for systemic therapy, different subsets of splenocytes from H2-K^b-Apoptin mice were evaluated. No deviation in B cells, helper-T or cytotoxic T cell populations was found compared to wild-type mice, signifying that Apoptin was not activated during any stage of their differentiation. The next question was whether these cells can still react properly to activating stimuli. Both transgenic and wild-type lymphocytes responded equally well to stimulation, indicating that the transgenic mice should be able to mount a proper immune response. The fact that Apoptin does not become active upon activation of T cells corroborates previous findings where ectopically expressed Apoptin in human T cells did not cause apoptosis after stimulation with phytohemagglutinin [4]. Furthermore, lysates from IL-2/conA stimulated T cells do not phosphorylate recombinant Apoptin, in contrast to lysates derived from tumor and transformed cells (ref.[15] and data not shown). Apparently, the signal transduction pathways used in normal stimulation and proliferation of T and B cells do not activate Apoptin.

The data presented here, demonstrating that it was not possible to detect negative effects of continuous Apoptin expression in lymphocytes *in vivo*, on the contrary, the cells appear fully functional, are an important first step for the development of systemic therapy. In the future, it will also be important to generate mice with a broader expression spectrum, to assess the effects of Apoptin in other organs. Meanwhile, the mice described here can be used to answer other important questions regarding Apoptin. Based on data generated so far, it cannot be excluded that Apoptin is active in tumor cells only when they are adapted to tissue culture, which selects a subpopulation of cells from the original tumor. A clear anti-tumor effect of Apoptin in tumors arising in the H2-K^b-Apoptin mice would eliminate the possibility that Apoptin reacts specifically to culture-induced changes. For this, Apoptin-transgenic mice should be crossed with a mouse strain prone to lymphoma. Simultaneously, that experiment will provide valuable information regarding the turning point in carcinogenesis that renders cells sensitive to Apoptin. Mice contracting lymphoma because of a specified mutation are in this case more informative than lymphoma induced by an applied carcinogen, which leads to a multitude of uncharacterised mutations. Finally, when established, this model can be used not only to study the mechanism of Apoptin, but also for molecular mechanisms that are currently difficult to study because of sporadic tumor formation originating from one in a million cells. This is for instance the case for studying the effects of c-myc on B cell development in Eμ-*myc* mice. In conclusion, we have

shown that it is feasible to generate Apoptin-transgenic mice. They are healthy and fertile, and the cells with the highest expression of Apoptin appear not to tolerate this well. The fact that the lymphocytes are not hindered in their development and function is an important prerequisite for systemic therapy with Apoptin.

Experimental procedures

Transgenic mice

To generate Apoptin transgenic mice the *Apoptin* gene was cloned into the first exon of the mouse H-2K^b promoter. The 450 bp BamH1 fragment derived from pCMV-vp3 [16] was inserted in the Not1 site of the pSB1 plasmid containing the HindIII-NruI fragment of the H-2K^b promoter [11], with the help of Not-BamH1 linkers (p21EcoA-vp3). Bacterial sequences were removed by EcoRI digestion and the 5.5 kb H-2K^b – *Apoptin*-EcoR1-restriction fragment was microinjected into the pronuclei of FVB fertilized eggs according to standard protocols [17]. Eggs that survived microinjection were implanted into the oviducts of pseudo-pregnant BCBA foster mice. From 300 transferable eggs implanted in 15 foster mice, 51 pups were born, of which 7 were transgenic. Two of these founders (E2 and D10) were backcrossed eight times in the standard FVB strain and also in the C57Bl/6 strain to exclude a strain-specific phenotype (Charles River, Lyon, France).

Genotyping of Animals

Southern analysis [18] was performed by fractionating *Bam*H1-digested DNA on a 1% agarose gel and blotting on to Hybond$^+$ (Amersham, UK). The blots were hybridised with randomly primed ^{32}P-labeled DNA fragment encoding Apoptin and results were obtained by autoradiography. PCR analysis was performed with high molecular weight DNA extracted from mouse tails with Dneasy tissue kit (Qiagen, Hilden, Germany). A 462-bp region was amplified with one oligonucleotide directed to the region in the p21EcoA plasmid prior to the *Apoptin*-start codon (5'ACCTGGGCCCAGCGGAGC-3') and one to the region after the *Apoptin*-stop codon (5'CGGATCCAACCCGGGTTGAA-3'). The presence of an amplified product indicates a transgenic genome. In brief, 1 μl DNA was added to 49 μl of 50 mM KCl, 10 mM TrisHCl (pH 8.3), 0.2 mg/ml gelatine, 1.5 mM $MgCl_2$, 0.1 mM of each deoxyribonucleotide, 20 pmol forward primer, 20 pmol reverse primer and 3 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, USA). PCR is performed for 30 cycles (94°C 30'', 54°C 60'', 72°C 60'') in an automated DNA Thermal Cycler (Perkin-Elmer Cetus). Sequencing was performed by Baseclear (Leiden, the Netherlands) with a sequence kit for GC-rich sequences using the PCR primers.

Cell culture and treatment

Osteosarcoma cells (Saos-2) and SV40-transformed fibroblasts (VH/SV) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (1% pen/strep, Life Technologies, Rockville, Md., USA) in a 5% CO_2 atmosphere at 37 °C. For immunofluorescence, cells were grown on glass microscope slides.

Splenocyte cultures were obtained by disaggregating spleen tissue aseptically through a 100 μm filter (Falcon, BD Biosciences) in complete DMEM medium. After centrifugation erythrocytes were lysed with NH_4Cl 8.4 g/l, $KHCO_3$ 1 g/l, pH 7.4 (Pharmacy LUMC, Leiden, The Netherlands) and cells were resuspended in spleen medium to a concentration of 1*10^6 cells/ml. Spleen medium consisted of 39% RPMI 1640 and 30% AIMV (both Life Technologies), 15% Fetal Clone I (Hyclone), 10% rat T stim (Becton Dickinson, Mountain View, CA), 2mM glutamax (Life Technologies), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 1% pen/strep, 4 μg/ml concanavalin A (con A, Pharmacia) and 10 U/ml IL-2 (Roche Diagnostics)). The proteasome was inhibited by addition of 5 μM clasto-lactacystin β lactone (Sigma, St. Louis, MO)) for 5 hours or 2 μM N-CBZ-Leu-Leu-Leu-al (Sigma) overnight to the medium.

B cell cultures were obtained by positive selection with αCD19 Dynabeads (Dynal Biotech, Hamburg, Germany) of splenocytes cultures after lysis of erythrocytes. After 20 minutes incubation B cells were selectively retained on an autoMACS column and resuspended in RPMI 1640 medium supplemented with 10% FCS and 1% pen/strep at 5*10^5 cells/ml. Cells were stimulated with lipopolysaccharide (LPS, Sigma) diluted to 5 μg/ml in the medium.

T cell cultures were obtained by removing B cells with magnetic Goat-anti-Mouse beads (Miltenyi Biotec, Germany). After 20 minutes incubation the beads were precipitated with a Promega-magnete, which was repeated twice. The remaining cells were resuspended in spleen medium at 1*10^6 cells/ml.

Plasmids and transfections

Expression plasmid pCMV-vp3 contains CAV DNA sequences encoding Apoptin (nt 427-868) under control of the cytomegalovirus promoter [16]. pCMV-desmin encodes desmin, a component of type II intermediate filaments, and was used as a negative control for the induction of apoptosis [19]. pHA-ubiquitin was a kind gift from dr. H. Masselink, NKI, The Netherlands. The ubiquitin is tagged with an epitope of the influenza hemagglutinin protein (HA).

Transfections were performed by means of the calcium-phosphate precipitation procedure as described by Graham and Van der Eb [20]. Briefly, 2 hours prior to transfection the medium of 30% confluent monolayers of osteosarcoma cells (Saos-2) or SV40-transformed fibroblasts (VH/SV) was replaced by fresh medium. DNA-calcium-phosphate precipitates were incubated for 30 min at room temperature, and subsequently added to the medium. The cells were washed twice with phosphate-buffered saline (PBS) after 24 hours and fresh medium was added.

Immunofluorescence and DAPI staining

Indirect immunofluorescence was performed as described previously [21]. To demonstrate the presence of Apoptin, cells were air dried and fixed with 80% acetone. The indirect immunofluorescence assay (IFA) was performed with hybridoma supernatant containing the mouse monoclonal antibody 111.3 [6] directed against Apoptin or a 25-fold dilution of the mouse monoclonal antibody 33 directed against desmin (Monosan, Uden, The Netherlands). Fluorescein-isothiocyanate-labelled goat anti-mouse antibody (Jackson Immunoresearch Laboratories, USA) was used as second antibody. Nuclear DNA was stained with 1 μg/ml 2,4-diamino-2-phenylindole (DAPI) in 2% 1,4 diazabicyclo-[2,2,2]-octane in glycerol/0.1 M TrisHCl pH 8.0.

Northern analysis

Total RNA was isolated from heart, thymus, muscle, brain, kidney, spleen, liver and lung from two H2-K^b-Apoptin mice according to the acid-guanidinium-phenol-chloroform protocol as described [22]. Twenty microgram RNA was run on a 1% agarose gel and blotted onto Hybond$^+$ membrane (Amersham, UK). Apoptin mRNA (2.3 kb) was hybridized with a randomly primed ^{32}P-labeled DNA fragment encoding Apoptin and visualized by autoradiography.

RNA *In situ* Hybridisation

Isolated organs were fixed overnight in 4% (w/v) formaldehyde in PBS. The organs were dehydrated in a graded ethanol series, paraffin-embedded, cut into sections, and mounted on aminoalkylsilane-coated slides. Serial sections were probed for the presence of Apoptin mRNA by in situ hybridization with a digoxigenin (dig) -labeled probe complementary to Apoptin mRNA. As a negative control, dig-labelled Apoptin mRNA was used. Probes were made according to the manufacturer's specifications (Roche, Mannheim, Germany). To obtain Apoptin RNA pobes (sense and antisense) the BamHI fragment of pCMV-VP3 was subcloned into pBluescript II SK-, either in sense or antisense orientation. Dig-labelled RNAs were transcribed by T7 RNA polymerase after linearization with XbaI.

Deparaffinated sections were washed twice with PBS and digested for 10 minutes at 37°C with 20 μg/ml proteinase K. After rinsing once with 0.2%glycine/PBS and twice with PBS, the sections were re-fixed for 20 min in 4% formaldehyde/0.2% glutaraldehyde dissolved in PBS and then washed twice with PBS. Sections were pre-hybridized for 1 hr and subsequently hybridized overnight at 70°C with approximately 300 ng/ml probe in 50% formamide, 5 x SSC, 1% block solution (Roche), 5 mM EDTA, 0.1% Tween-20, 0.1% Chaps (Sigma), 0.1 mg/ml heparin (Becton-Dickinson), and 1 mg/ml yeast total RNA (Roche). Sections were subsequently rinsed in 2 x SSC, pH 4.5, washed twice at 65°C in 50% formamide/2 x SSC, pH 4.5, followed by three washes with PBS/0.2 %Tween. Probe bound to the section was detected using sheep anti-digoxigenin Fab fragment covalently coupled to alkaline phosphatase and NBT/BCIP as chromogenic substrate, according to the manufacturer's protocol (Roche). Sections were washed with double-distilled water, dehydrated in a graded ethanol series and xylene, and embedded in Entellan (Merck, Darmstadt, Germany).

Immunohistochemistry

Paraffin-embedded sections of spleen and thymus tissue obtained from H2-K^b-Apoptin mice and wild-type littermates were stained for Apoptin by a three-step immunoperoxidase staining using a polyclonal rabbit antibody recognising the C-terminus of Apoptin, αVP3C (produced by EurogenTec, Seraing, Belgium) as described[10].

Immunoprecipitation and western analysis

Splenocytes were isolated as described earlier under 'cell culture and treatment'. Isolated lung was flash-frozen in liquid nitrogen and subsequently grinded with a microdismembrator. Live spleen cells or frozen lung powder were resuspended in lysis buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.1% Triton and protease inhibitors) and incubated on ice for one hour. The lysate was cleared by centrifugation and immunoprecipitated with Protein A-sepharose beads

(Sigma-Aldrich, Zwijndrecht, The Netherlands) coupled to a polyclonal rabbit antibody recognising the C-terminus of Apoptin, αVP3C or to the mouse monoclonal DO-1 recognizing p53 (Santa Cruz biotechnology, Santa Cruz, CA). The immunoprecipitates were washed three times with cold lysis buffer and boiled for 5 min in Laemli sample buffer. Samples were subjected to electrophoresis on a 12.5% polyacrylamide SDS gel, followed by transfer to Immobilon P membranes (Millipore, Bedford, Massachusetts, USA). After blocking in milk buffer (5% milk in Tris-buffered saline with 0.2% Tween (TBST)) the membranes were incubated with the mouse monoclonal antibody 111.3 against the N-terminus of Apoptin, HA11 directed against the hemaglutin-tag on transfected ubiquitin (Babco, Berkeley, CA, USA) or DO-1 against p53 when appropriate. The membranes were washed with TBST, and proteins were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) secondary antibody, followed by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, UK).

Flow cytometry analysis

Spleen tissue was disaggregated through a 100 μm filter (Falcon) in complete DMEM medium. After centrifugation for 8 minutes at 800 rpm, single cell suspensions were washed in cold phosphate buffered saline (PBS) containing 0.5% BSA. $5*10^4$ cells were directly labelled for 30 min using αCD19-Fitc, αCD3-Fitc, αCD4-APC, αCD8-APC antibodies (BD PharMingen, UK) to detect B cells, T cells, helper T cells and cytotoxic T cells, respectively. Samples were analysed on a FACScalibur (BD Pharmingen).

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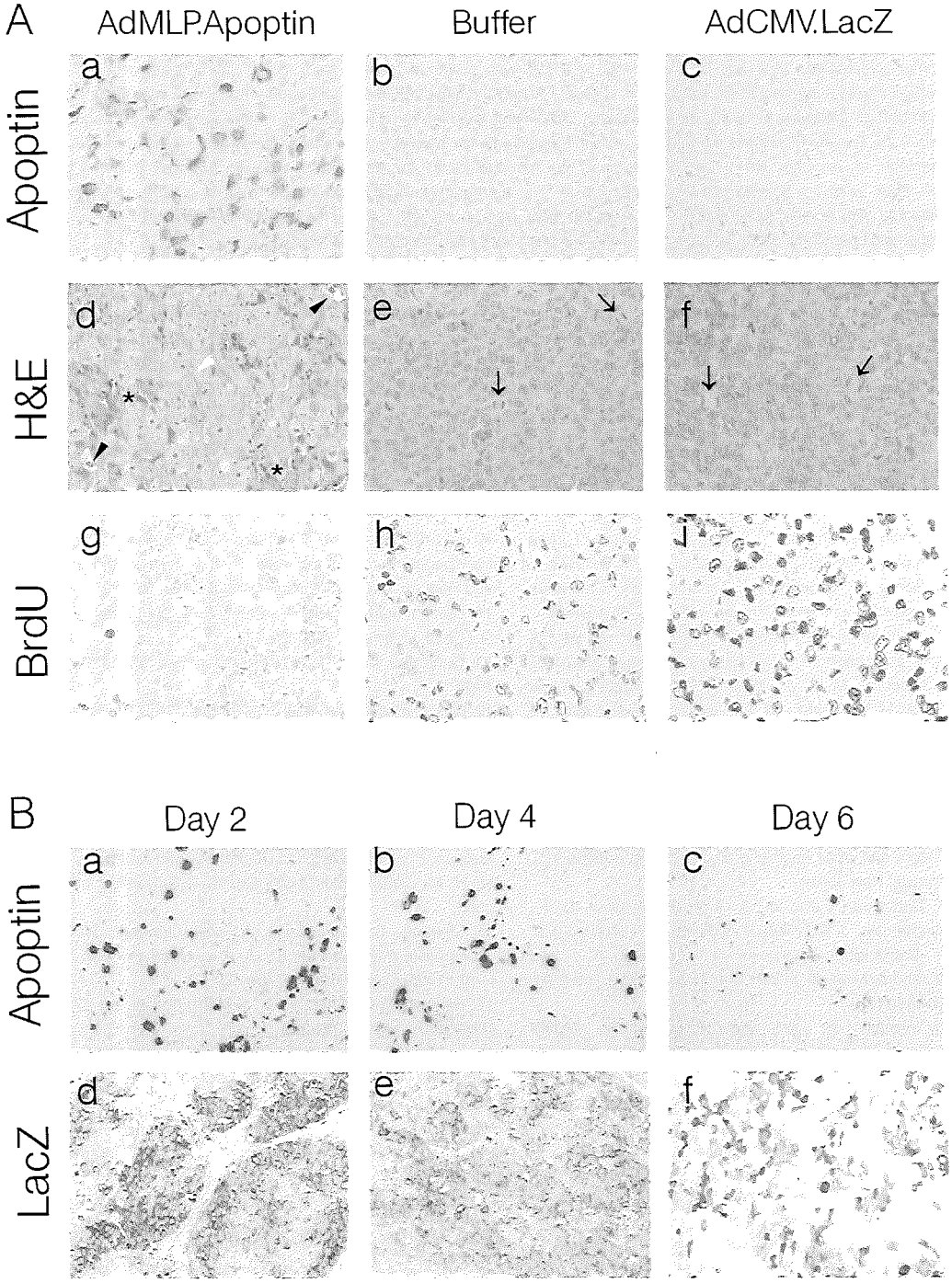

Figure 2B From chapter 3 page 70. Legend see page 71.

Chapter 6

Summary + discussion

Index:

Chapter 6. Summary + discussion

The chicken anaemia virus was cloned in 1990 and analysis of its three proteins led to the serendipitous finding that viral protein 3, or Apoptin, induces apoptosis in many cultured human tumour cells but not in their normal counterparts. The ensuing question, whether the activity and specificity of Apoptin would also hold true *in vivo*, was addressed in the research described in this thesis. Besides preliminary confirmation of this concept, my studies have yielded some important guidelines for the development of an anticancer therapy based on Apoptin gene or protein therapy. Moreover, the full potential to use Apoptin as an anticancer agent is intimately linked with the unravelling of its mechanism of action. These data will have implications for the safe application of Apoptin, the feasibility of developing small molecular drugs, and the suitability for combination therapies, as will be discussed below.

Apoptin as a therapeutic agent

Adenovirus vectorology

To exert its anti-tumour activities in humans, the chicken virus-derived Apoptin has to be introduced into cells *in vivo*. An efficient way to achieve this is by employing mammalian viruses as carriers. Chapter 2 describes the generation of a replication-deficient adenovirus expressing *Apoptin*. At the moment of generation, the safety issues of replication-competent viruses in gene therapy were still under heavy discussion, and since we were, to begin with, looking for a proof of principle of the *in vivo* anti-tumour activity of Apoptin itself, we chose this well-studied, non-replicating virus as a delivery vector.

Production of viral vectors containing genes toxic to packaging cells has proved difficult, illustrated by the problematic attempts to generate adenoviruses expressing FasL and Bax [1,2]. Fortunately, apoptosis induction by Apoptin is relatively slow and as the replication cycle of recombinant adenoviruses *in vitro* takes about 32-48 hours, we were able to demonstrate that the induction of apoptosis by Apoptin in the transformed helper cells occurred predominantly after these first two days, rendering the production of Ad-*Apoptin* technically feasible.

Eventually, when preclinical data with an adenovirus expressing *Apoptin* would warrant clinical testing, the risk of co-injecting replication-competent viruses must be restricted to a minimum. Adenoviral vectors are rendered replication-deficient by deletion of the E1 region from their genome. Producer cell lines, such as 293, 911 and PER.C6, have the adenovirus E1 region integrated in their genome to allow the replication of E1-deleted adenoviral vectors. The first generation vector AdMLP.*Apoptin* contains a short stretch of adenovirus sequence that is also present in the producer cells. Potentially, replication-competent adenoviruses (RCA) could arise from non-replicating vectors through regaining the E1 sequence from the helper cells by homologous recombination [3]. Although all of the batches we produced were found negative for RCA with a PCR assay that detects one RCA particle among ten million E1-deleted particles, the

risk of RCA increases when the scale of production is enlarged. Therefore, two new adenoviral vectors expressing *Apoptin* have been generated by Ronald Vogels at Introgene (presently Crucell, Leiden), called AdCLIP.*Apoptin* and AdApt.*Apoptin*, both expressing *Apoptin* under the regulation of the CMV promoter and with viral backbones that are perfectly matched to the PER.C6 cell line, thereby preventing the emergence of RCA through homologous recombination (chapter 4).

Multiple large-scale batches (in 3-10 liter fermentors) of all three *Apoptin*-expressing adenoviruses have been produced by George Roth at Berlex Biosciences (Inc., Richmond, CA, USA), to be used in preclinical studies. Since large-scale production and standardization under GMP (good manufacturing practice) are prerequisites for clinical testing as well, these productions provide important information on the technical feasibility of an Apoptin-based medicine using these vectors. In small-scale productions, AdMLP.*Apoptin* exhibited similar kinetics and yields as control viruses (chapter 2). In addition, no differences were observed for AdMLP.*Apoptin*, the new vector AdClip.*Apoptin* and their anti-sense counterparts when subjected to large-scale virus production. However, the second CMV-vector, AdApt-*Apoptin*, yielded fewer infectious particles than did AdMLP.*Apoptin,* AdClip.*Apoptin* or the control viruses. Nevertheless, AdApt.*Apoptin* cleared dishes of HepG2 and MDA breast cancer cells at a lower multiplicity of infection (m.o.i.) than did AdClip.*Apoptin* (D. Henderson and G. Roth, personal communication). This led us to speculate that the expression of *Apoptin* by AdApt might be earlier or higher than that of the other constructs. If this increased expression would lead to cell death prior to virus particle formation in a higher percentage of the packaging cells, this could explain the apparently lower yield observed in the large-scale productions, as judged by total infectious particles (titer times volume). However, such premature cell death would consequently give rise to an underestimation of the titer (amount of infectious particles per millilitre), since some cells in the plaque assay would also perish before they could infect their surrounding cells, thus resulting in fewer plaques than actual infectious particles. This putative titer underestimation could explain the apparent lower virus dose of AdApt.*Apoptin* required to kill tumour cells, i.e. the actual dose would then have been comparable to the other constructs.

To distinguish whether the effects of AdApt.*Apoptin* versus the control, non-expressing, AdApt.*Antisense* in preclinical studies are attributable to Apoptin, as opposed to differences in viral dose, a new assay for titration of adenoviral-vectors expressing *Apoptin* should be developed, one based solely on infection of cells and not on subsequent lysis and re-infection of neighbouring cells. Preliminary data based on DNA quantification suggest that the titer of AdApt.*Apoptin* may be underestimated by a factor of 5-10 (S. Rutjes, unpublished data). For Fas-expressing adenoviruses an underestimation of 2000-fold has been described [2]; the less dramatic titration effect of Apoptin in the AdApt vector and the absence of detectable effects in the other vectors is most likely due to its relatively slow induction of apoptosis. To investigate further the differences in viral production and anti-tumour effects of the AdApt vector *versus* AdMLP and AdCLIP, the timing and levels of Apoptin expression by the different constructs in the packaging cell lines could be compared to the expression of early and late viral gene expression.

Fortunately, no difference in yield was observed between AdMLP.*Apoptin* and control vectors, suggesting that in this context Apoptin did not affect the viral production to noticeable extent; therefore the conclusions that the anti-tumour effects described in chapter 2 and 3 with AdMLP.*Apoptin* are due to Apoptin remain the most plausible. Nevertheless, the findings with AdApt.*Apoptin* stress the importance of careful characterization of viral vectors expressing apoptotic genes, as this could have major implications for the interpretation of data generated with those.

In addition to these considerations for the titration of viral vectors carrying toxic genes, it should be noted that large-scale productions of such vectors are at risk of generating non-functional mutants. Specifically, even though the majority of virus particles is assembled prior to the toxic effects of Apoptin in transformed producer cells, a subset of cells (approximately 20%, see chapter 2) is killed by Apoptin prior to viral production, providing a window for negative selection against wild-type Apoptin. In fact, several rounds of propagation with successive virus batches (versus always starting from the initial mother stock) has resulted in an AdMLP.*Apoptin* virus batch that expressed mutated Apoptin that was atypically distributed over the nucleus and cytoplasm and did not induce apoptosis in tumour cells (A. Pietersen and J. van Tongeren, unpublished results). Besides the fact that analysis of this mutant batch may provide valuable insights into the mechanism of Apoptin, this observation also indicates that caution should be taken with large-scale productions, as expanding the number of infected cells increases the chance of attenuating mutations.

Recently, an innovative approach has been developed that provides a solution for the potential problems of lower yield and titer underestimation, as well as the risk of selecting mutants. Gu and coworkers make use of the Tet-Off system in a bicistronic adenoviral vector for the generation of a Bax-delivery method. The virus expresses a transcriptional transactivator (tTA), which can induce the synthetic promoter that regulates the *Bax* gene present in the same viral construct. However, when tetracycline is added to the medium of the viral packaging cells, the transactivator will be inhibited, thereby preventing *Bax* expression. Viral production can occur without Bax-mediated toxicity to the packaging cells, but when the viruses are used to infect tumour cells, the absence of tetracycline will result in robust *Bax* expression and subsequent tumour cell death [4]. Inclusion of the *Apoptin* gene in such a vector would exclude potential effects of Apoptin expression on the viral producer cells and it may therefore be advisable to use this system for large-scale productions of adenoviral vectors expressing *Apoptin* when clinical testing is pursued.

Apoptin in other viral vectors

Adenoviral vectors infect a broad range of cell types, but are not very effective in transducing haematopoietic cells. To test Apoptin as a therapeutic agent for haematopoietic malignancies, a vector that does infect these cells is required, such as certain recombinant retroviruses, like Moloney murine leukaemia virus (MoMLV) or human immunodeficiency virus (HIV). Several

attempts have been made to generate these RNA-based viral vectors expressing *Apoptin*, but so far these have not been successful (Olijslagers and Rutjes, manuscript in preparation). In the case of the MoMLV-based vector, infection of tumour cells with supernatant derived from producer cell lines with stable integrated *Apoptin* constructs did not result in Apoptin expression in the malignant cells. Transient transfection of the constructs of either the MoMLV or the lentiviral system to generate Apoptin-expressing retroviruses in a batch-wise fashion, in contrast to the stable integration method, did not improve this. On one hand, it seems that Apoptin expression is detrimental for the generation of these vectors, as cotransfection of an *Apoptin*-expressing plasmid with plasmids for the generation of a GFP-lentivirus significantly reduced viral production, whereas this was not the case with an empty-control plasmid (S. Rutjes, personal communication). Because retrovirally-infected cells do not lyse, as they do with adenoviral infection, but particles are instead continuously exocytosed from the cytoplasm, it may be that the retroviral production is hindered by Apoptin-induced apoptosis in the transformed producer cells. It can also be that Apoptin directly interferes with the replication cycle of retroviruses. However, with the lentiviral approach, *Apoptin*-containing particles are formed, albeit at a lower level, and the lack of Apoptin expression following subsequent infection may be due to low expression levels by retroviruses. Since only one or a few viral particles may integrate and express *Apoptin*, the level of Apoptin protein per cell may remain under the detection limit of the antibodies used in Western Blot and immunofluorescence analysis. This theory is supported by preliminary experiments using proteasomal inhibitors that reveal the presence of some Apoptin protein after lentiviral infection of HeLa cells (S. Rutjes, personal communication). The Apoptin expression level may influence the suitability of delivery vectors, as is discussed below in the section 'Intracellular Apoptin levels'.

A viral vector that is also capable of infecting haematopoietic cells, is the autonomous adeno-associated virus called Parvovirus. This virus, which has a DNA genome, proved compatible with Apoptin expression and gave rise to high Apoptin protein levels. Parvoviruses already hold some intrinsic tumour-selectivity (see chapter 1), but are not always potent enough to cause eradication of all tumour cells. Incorporation of the *Apoptin* gene in the place of the genes encoding the capsid proteins, together with the tumour-selective expression of the toxic NS-1 protein of the parvovirus, extended the range of tumour cells sensitive to parvovirus in cell culture [5]. If future experiments demonstrate that Apoptin is also compatible with replication-competent parvoviruses, this parvoviral vector system would constitute an alternative approach for cancer gene therapy with Apoptin, although more knowledge on the biology of the parvovirus and its proteins would be desirable.

Transduction in vivo

The generation of the adenoviral vector expressing *Apoptin* enabled us for the first time to examine the effects of Apoptin *in vivo*. A pilot experiment described in chapter two showed a delay in tumour growth seven days after a single injection of AdMLP.*Apoptin* in xenografted

human hepatoma. We also noted that these tumours exhibited lobular growth and that a single injection was often confined to one such lobule, thereby impairing transduction of the rest of the tumour. To increase the transduction efficiency, we used a regimen of multiple injections over a period of several days in the large-scale, long-term experiment described in chapter 3. This approach resulted in a significant overall survival benefit for the Apoptin-treated mice and in some cases, complete regression of the established tumour. Our data demonstrating the *in vivo* anti-tumour effect of Apoptin have been corroborated by preclinical studies at Berlex Biosciences (Inc., Richmond, CA, USA, D. Henderson, H-L. Liu, G. Roth and M. Halks-Miller, unpublished data). There, nude mice bearing human breast carcinoma (MDA231MT cells) were treated with eight injections of AdApt.*Apoptin* or AdApt.*Antisense*, compared to five doses of taxol. Twenty-one days after the start of the treatments, both the Apoptin gene therapy and the taxol chemotherapy had prevented tumour growth, whereas the tumours treated with vehicle or antisense-viral vector continued their exponential growth (figure 1). Even if the titer of AdApt.*Apoptin* had been underestimated, treatment with Apoptin proved as efficient as the lowest dose of taxol and had none or only minor effect on the weight of the treated mice,

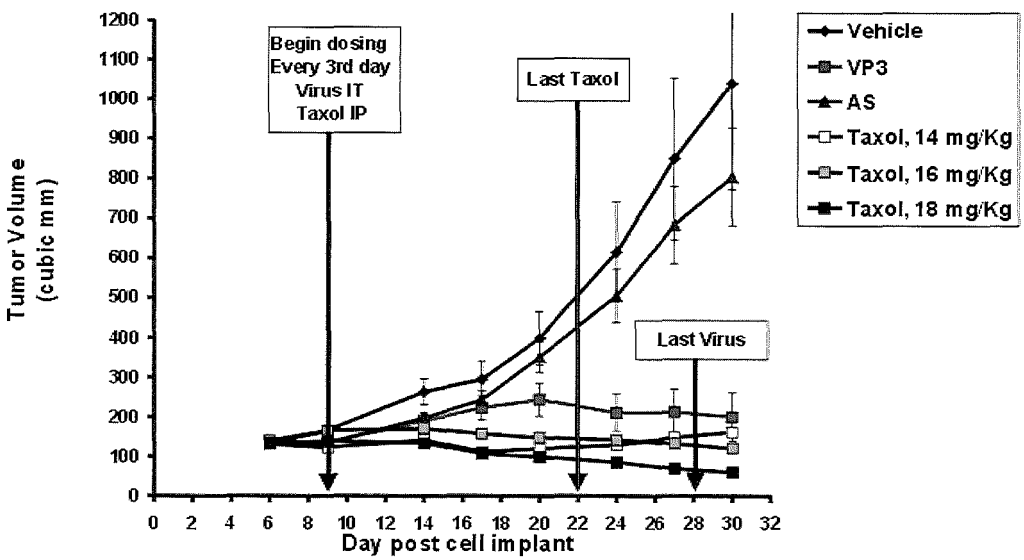

Figure 1. Effect of AdApt.Apoptin and chemotherapy on human breast carcinomas xenografted on nude mice.

Animals bearing MDA2331-MT1 tumours received $2.1x10^9$ plaque forming units (pfu) of AdApt.*Apoptin* or AdApt.*Antisense* intratumourally every third day for a total of 8 doses starting 9 days after tumour implantation. Control animals received either vehicle or 5 doses of taxol at 14, 16, and 18 mg/kg intraperitoneally. Tumour volume was measured twice a week until the study was terminated at day 31. Apoptin expression was detectable in a small percentage of tumour cells at the end of the study as determined by immunohistochemistry. Experiments were performed at Berlex Biosciences.

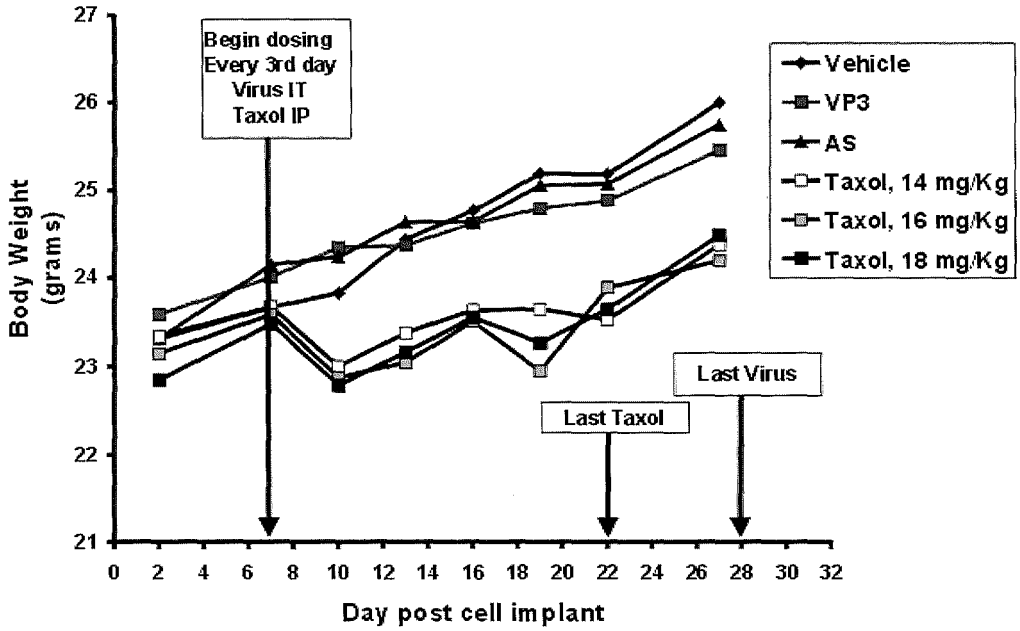

Figure 2. Effect of AdApt.Apoptin and chemotherapy on the body weight of mice bearing MDA231-MT1 tumours.

Body weight of the mice (whose tumour volumes are depicted in figure 1) as a crude measure for toxicity of the treatment with taxol and the adenoviral vectors expressing Apoptin. Experiments were performed at Berlex Biosciences.

whereas the chemotherapeutic regimen resulted in life-threatening weight loss (figure 2). The local administration of the Apoptin-adenovirus *versus* the systemic treatment with taxol precludes a comparison of toxicities, but it does demonstrate the effectiveness of Apoptin expression *in vivo*.

With successful preclinical results achieved, it became necessary to choose an appropriate clinical model. Carcinoma of the bile duct formed an attractive candidate. This cancer is insensitive to conventional cancer therapy, probably because tissue-specific damage (e.g. bile salts) leads to potent blockage of the pathways utilized by chemotherapeutics and radiation. Strikingly, Apoptin was able to induce apoptosis in three independent cholangiocarcinoma cell lines irrespective of their individual mutations (chapter 4), further supporting the model that Apoptin acts downstream in the apoptotic pathway and indicating that current therapy-resistant tumours might benefit from an Apoptin-based therapy. However, clinicians disagree on the feasibility of using a replication-deficient vector for the treatment of cholangiocarcinoma. We already observed limited viral spread in our hepatome xenograft studies, and even after a regimen of multiple injections over several days, there was considerable variation in the response of individual tumours, probably due to differences in transduction. Biliary tract cancers

comprise several cell layers and can be located in bile ducts embedded in the liver, rendering them difficult to reach. Presumably, injection of replication-deficient adenoviruses into the bile duct would only transduce the outer layer of cholangiocarcinoma cells [6]. Although it has been postulated that semi-local administration by occlusion of the bile ducts with dotter-like balloons, potentially accompanied by intratumoural injections, might improve the transduction efficiency, the predominant notion seems to be that gene therapy with a non-replicating vector transducing a therapeutic protein without bystander effect will be ineffective. Therefore, despite the promising potency of Apoptin in these therapy-resistant carcinomas, the non-replicating adenoviruses expressing *Apoptin* will not be tested in clinical trials for cholangiocarcinoma.

Replicative-deficient viruses carrying transgenes such as p53 or HSV-TK have been useful in providing the first proofs of principle for gene therapy of cancer in the clinic, and although modest successes with these vectors have been reported, most attention is currently focussed on selectively-replicating vectors, as these have proved to be relatively safe, showed superior spread within the tumours and have yielded impressive results in phase III clinical trials (see introductory chapter). Similarly, the preclinical studies with replication-deficient adenoviruses expressing *Apoptin* have been essential to validate the concept of Apoptin as an anticancer agent. They have demonstrated that when Apoptin is expressed in a tumour cell *in vivo* it will kill it, and that under optimal delivery circumstances entire tumours can be eradicated. However, the data also demonstrate the current inefficiency of transduction, and therefore, improvement of the delivery and/or bystander effects of Apoptin are warranted before any clinical trial can be initiated.

Enhancing transduction efficiency

In cancer gene therapy, especially against disseminated disease, one of the largest problems constitutes the targeting of each and every tumour cell. As illustrated by the previous section, a therapy based on Apoptin is also dependent on the efficiency of transduction. Besides engineering more efficient delivery vehicles, evoking a 'bystander effect' could also substantially increase the efficacy of therapies based on toxic genes. The term 'bystander effect' was traditionally used to describe the effects of prodrug therapy, but it can be more broadly applied to processes that affect the non-transduced cells, such as anti-angiogenesis or protein transport.

Presently, no clear bystander effect has been detected with Apoptin. The small scale experiment described in chapter 2 showed some remarkable effects on the vascularisation of certain tumours, which could be due to a direct effect on angiogenesis, or by an indirect effect of diminishing angiogenic factors caused by disappearance of tumour cells. However, the effect on tumour vasculature was much less pronounced in the study described in chapter 3. An experiment specifically designed to measure the effect of Apoptin on angiogenesis could provide further evidence and insight for the provocative initial observation.

In recent years, considerable effort has been put into approaches to actively endow a bystander effect on Apoptin. One approach was based on fusing Apoptin to the herpes simplex virus protein VP22. Others have shown that VP22 migrates from the initial transfected cells to surrounding cells, ultimately transducing an entire culture dish [7]. When a VP22-p53 fusion was transduced by an adenoviral vector, VP22 mediated intracellular transport of p53 into normal livers and liver tumours in mice, increasing the anti-tumour activity compared to a virus expressing p53 alone [8]. Apoptin was fused to both the N- and C-terminus of VP22, but unfortunately the fusion proteins were not transported to neighbouring cells after transfection (Rutjes, in preparation). In fact, the VP22-Apoptin products seemed to reside in aggresome-like structures in transfected cells and were almost exclusively present in the insoluble fraction of cell lysates. Another protein transduction strategy is based on the HIV-derived TAT-peptide. When injected intravenously, TAT-fusion proteins transduced virtually all tissues of mice, even crossing the blood-brain barrier [9]. Again, the biochemical properties of Apoptin proved extremely unfavourable for the production of a TAT-Apoptin fusion protein, both in our lab (M. Voorhoeve and K. Kooistra, unpublished results), as well as in the lab that first developed the TAT peptide (S. Dowdy, personal communication). An attempt to optimise this process could comprise deleting the part of Apoptin that causes the insolubility. If such an approach would indeed be successful in transporting Apoptin, while retaining its tumour-specific activity, the resultant ability to deliver Apoptin systemically to all tissues would greatly enhance the potential of Apoptin for cancer therapy.

Another option currently being explored to enhance the local delivery/intratumoural spread is the inclusion of Apoptin into conditionally-replicating viral vectors. This combination may ensure enhanced spread of Apoptin throughout the tumour, and Apoptin could add potency to these vectors, which for unclear reasons are not able to lyse an entire tumour on their own [10]. Apoptin may even function as an Adeno Death Protein (ADP), improving viral release in a tumour-selective fashion. Very late in the wild-type adenoviral replication cycle, when viral particles have formed in the cell nucleus, ADP is expressed to facilitate release of the adenoviral particles. Viral vectors selectively replicating in cells lacking RB are more cytolytic when they are genetically modified to overexpress ADP [11]. If apoptosis induced by Apoptin would be equally efficient in mediating viral release, it would add an additional safety feature in that viral spread would be enhanced only from tumour cells, and not from normal cells. In this way, systemically administered virus is predicted to have even lower side effects. Obviously, production of such viruses needs to be carefully monitored for effects of Apoptin on viral yield and titration assays to exclude the generation of mutants. Importantly, the kinetics of Apoptin-induced apoptosis may be too slow to have an effect on viral release, or the induction of cell death may even be incompatible with the viral burst mechanism, rendering the inclusion of Apoptin in these vectors ill-suited in both cases. Hence, appealing as the concept may be, experiments are required to demonstrate its feasibility.

Apoptin in healthy cells

Toxicity studies

Toxicity to non-tumour cells represents a major determinant of the therapeutic window, and thus of treatment success, particularly in the case of systemic delivery. The fact that cell types damaged by classical cancer therapies, such as $CD34^+$ stem cells and hepatocytes, are not sensitive to Apoptin *in vitro* is encouraging [12], Zhang, unpublished observations).

Reassuringly, the inactivity of Apoptin in normal cells has been independently confirmed by Berlex Biosciences, in experiments in which they infected normal cell cultures of dermal fibroblasts, mammary epithelial cells and endothelial cells with AdApt.*Apoptin* or AdCLIP.*Apoptin* (H. Dinter, unpublished data). Of note, primary human fibroblasts or rat hepatocytes infected with AdApt.*Apoptin* in our lab exhibited nuclear expression of Apoptin, however, this did not lead to an increase in apoptosis (S. Rutjes, unpublished observations). Furthermore, transfection of primary, $CD31^+$ cell-depleted fibroblast cultures with Apoptin-expressing plasmids in the research laboratories of Schering AG (Berlin, Germany) again corroborated our findings that Apoptin localises in the cytoplasm and does not induce apoptosis (D. Mumberg, personal communication). However, all *in vitro* studies are limited by the short life span of primary cells in culture, which obstructs any long-term evaluation of the effects of Apoptin in normal cells. Thus, because not all toxic effects can be predicted from *in vitro* studies, the validation of Apoptin as an anticancer agent relies heavily on preclinical studies.

I have examined the effects of Apoptin in normal cells *in vivo* after adenoviral overexpression (chapter 2) and after continuous expression throughout development in transgenic mice (chapter 5). The first *in vivo* toxicity experiment comprised the injection of AdMLP.*Apoptin* in rats and demonstrated that subcutaneous, intraperitoneal and even intravenous administration did not result in toxic effects. Later on it became apparent that the major late promoter may be less active in rodent cells than in human cells [13]. In cultured rat hepatocytes, Apoptin could be visualized by immunofluorescence after infection with AdMLP.*Apoptin*, showing clear cytoplasmic localisation and no induction of apoptosis during their limited life-span in culture (chapter 2). Unfortunately, at the time of the *in vivo* toxicity study, we had not yet solved the technical difficulty of detecting Apoptin protein in tissue sections. After publication, the detection of Apoptin in paraffin sections was enabled by a new antigen-retrieval protocol (chapter 3). Subsequent staining of the liver sections from our toxicity study failed to detect Apoptin expression, even though it was demonstrated that the livers of these rats were efficiently infected (as assessed by the detection of viral DNA in this tissue) and that in the same procedure HepG2 tumour tissue infected with AdMLP.*Apoptin* was positive (S. Rutjes, unpublished observations). It could be that the MLP promoter of this vector was not active *in vivo*, or that the Apoptin levels remained below detection level due to a possible normal-specific instability (see section below on intracellular Apoptin levels). Both possibilities are contradictory to the *in vitro* experiments that suggest that substantial Apoptin protein levels can be achieved in rat hepatocytes after infection

with AdMLP.*Apoptin.* This discrepancy prevents definite conclusions over the absence of adverse effects. Therefore, the use of our newly generated adenoviral vectors that express *Apoptin* under the regulation of the CMV promoter, which should work equally well in rodent and human cells, should allow a more definitive *in vivo* toxicity study.

Apoptin-transgenic mice

Previous work on the tumour-specificity of Apoptin has understandably focussed on human cells. However, performing studies in rodents to obtain long-term toxicity data requires that Apoptin's behaviour is examined in rodent cell cultures as well. Importantly, Apoptin was shown to induce apoptosis in rat CC531 and mouse B16 tumour cells (A. Pietersen, unpublished results), but not in rat hepatocytes (in cell culture, where Apoptin expression was detectable) and most likely also not in mouse lymphocytes (chapter 2 and 5). These observations imply that Apoptin is also selectively active in rodent cells, in contrast to, for instance, E4orf4. Together with the absence of apoptosis when Apoptin is expressed in mouse embryo fibroblasts (Y-H Zhang, personal communication), these results render transgenic mice suitable preclinical models for Apoptin.

As discussed in the introductory chapter, several approaches exist to deliver toxic genes to tumour cells. The more specific the gene, the less targeted the approach needs to be. For the development of Apoptin as an anticancer agent it is therefore of paramount importance to know whether there are any normal cell types that are sensitive to Apoptin at any time of their life-span. The identification of such populations could contribute to the choice of delivery vehicle so that these cells are not transduced and toxicity is kept to a minimum. If that delivery system does not exist or is incompatible with Apoptin, the information can be used to predict contra-indications and perhaps, what cancer types would be suitable for Apoptin treatment.

The results from the H2-K^b-*Apoptin* mice appear promising for systemic treatment with Apoptin regarding the lack of effects on the immune system. Nevertheless, before any definite conclusions can be drawn, this model must be validated by assessing the activity of Apoptin in these cells when they transform into lymphoma. Probably the most straightforward way to test this is by crossing the *Apoptin*-transgenic mice with mice that have an increased incidence of lymphoma, for instance p53-null or Eμ-myc, INK4A$^{+/-}$ transgenic mice. If the level of Apoptin expression that we observed in transgenic lymphocytes (chapter 5) is capable of triggering apoptosis once these cells become transformed, the cancer-prone mice that also express *Apoptin* should exhibit a significant delay in occurrence of lymphoma. A complete protection against lymphoma is not to be expected, since there will be selection pressure against Apoptin expression. Specifically, the presence of the Apoptin-transgenic locus will force an additional 'hit' that needs to occur in addition to the acquisition of the other cancer hallmarks; therefore, the stochastic inactivation of Apoptin should add substantial time to the latency of the lymphoma. If this delay is indeed observed, the experiment shows that the Apoptin levels are sufficient and sanctions the conclusion that Apoptin is not toxic after prolonged expression in B and T cells *in*

vivo. After validation, the *Apoptin*-transgenic mice additionally constitute a mouse model that can be used to study the effects of proto-oncogenes on normal cell populations, something that is currently not possible. For example, the analysis of the effects of c-myc on the development of B cell compartment is obstructed by the rare emergence of a tumourigenic B cell that will rapidly populate the haematopoietic system and become lethal to the mouse. Crossing these mice with the H2-K^b-*Apoptin* mice could provide valuable information on the regulatory role of c-myc in normal cell processes because, due to elimination of the occasional transformed cell, the normal B cells can be studied for a longer period.

Most importantly, however, validation of the *Apoptin*-transgenic mice would prove that Apoptin is also active in tumours that arise *in vivo*. The majority of tumours isolated from patients fail to proliferate when explanted *in vitro;* in fact, it has been estimated that less than 1% of tumours can be subjected to continuous cell culture. Due to technical constraints, we have not been able to test Apoptin in freshly isolated tumour biopsies; hence the activity of Apoptin has been tested solely in *in vitro*-transformed cells and in tumour cell lines that have been selected to grow in culture. Although it has been shown that freshly isolated biopsies exhibit increased Apoptin-kinase activity [14], it can not yet be excluded that Apoptin responds to changes induced by tissue culture in the tumour cells, in combination with their oncogenic phenotype. Therefore, demonstrating an anti-tumour effect of Apoptin in tumours arising in transgenic mice would be an important step toward clinical testing.

Additionally, *Apoptin*-transgenic mice can be used to obtain insight into the circumstances that lead to the activation of Apoptin. By inducing tumorigenesis in these mice, one could identify the 'turning point' when a normal cell becomes sensitive to Apoptin. It has been shown that normal fibroblasts from cancer-prone individuals, e.g. carriers of germline tumour-suppressor mutations, are not sensitive to Apoptin. However, after a pulse of UV-radiation Apoptin does migrate to the nucleus and induces apoptosis, whereas fibroblasts from healthy individuals remain resistant to Apoptin even after UV exposure [15]. These results suggest that loss of one p53 allele or mutation of both p16 copies, which were the characteristics of two of the cancer-prone syndromes, are not sufficient to activate Apoptin. Crossings of *Apoptin*-transgenic mice with genetically-defined cancer-prone mice for instance overexpressing c-myc or Ras, lacking p53 or p16, or harbouring other tumorigenic alterations can elucidate whether Apoptin reacts to any of these cancer characteristics alone or in particular combination. Part of the question can be addressed *in vitro*, since transgenic mice allow the isolation of a primary cell culture that generally expresses Apoptin. Transduction of primary cells *in vitro* can be extremely inefficient; therefore, the transgenic cells constitute an attractive tool for biochemical studies on the effect of Apoptin in normal cells.

Furthermore, the generation of an *Apoptin*-transgenic mouse with a strong, broad-range expression such as that directed by the actin promoter or the Rosa locus would constitute an effective means to study whether there are cell types besides the lymphoid compartment that are sensitive to Apoptin at any phase of their life-span. If some cells are indeed found to be sensitive to Apoptin, these data could provide additional clues for the mechanism of action of Apoptin.

High levels of Apoptin expression in colon or skin of these mice would allow analysis of well-characterized models for carcinogenesis in these tissues. The spectrum of lesions found in Apoptin-transgenic mice may for instance be predominantly restricted to hyperplasia while neoplasia occurs frequently in their wild-type littermates. These studies could thus contribute further to determine at what stage of transformation Apoptin becomes activated.

Intracellular Apoptin levels

An important question that is currently being addressed is whether there is a difference in Apoptin's activities when it is overexpressed by, for instance, transient transfection or adenoviral infection compared to relatively low expression from a few integrated copies as is the case with transgenic mice and retroviral infections (S. Rutjes). If a considerable amount of Apoptin is necessary per cell to cause apoptosis, this requirement will restrict the choice of treatment modality for Apoptin. Experiments titrating down an *Apoptin*-expressing plasmid suggest that such a threshold level indeed exists (thesis Olijslagers), further emphasising the importance of validating that the H2-K^b-*Apoptin* mice express sufficient protein to exceed this threshold.

An intriguing possibility is that the stability of Apoptin protein may be differentially regulated in normal versus malignant cells. Namely, the observation that Apoptin expressed in healthy transgenic cells is unstable is in keeping with data from microinjections of recombinant Apoptin in normal cells. Here, Zhang and colleagues showed that early after injection, the biochemical properties of Apoptin in normal cells differ from that in tumour cells with respect to the epitope for an Apoptin-specific antibody, which becomes inaccessible in normal cells only [12]. After stringent lysis, this epitope is retrieved, indicating that the protein is still present. However, somewhat later, the recombinant Apoptin disappears from the normal cells. By then, all tumour cells have already undergone apoptosis, precluding a comparison of the half-life of Apoptin in tumour and normal cells. Experiments designed to compare the stability of Apoptin between tumour and normal cells are necessary to determine the relevance of the degradation of Apoptin in normal cells for its tumour-specific actions. This information may again be significant for choosing a suitable delivery vector. If Apoptin is indeed rapidly degraded in normal cells, this would have implications for the applicability of, for instance, the TAT-Apoptin fusion. In this system, the fusion protein will cross cell membranes until equilibrium is reached [9]. If TAT-Apoptin is continuously degraded in normal cells, the entire body (excluding the tumour) would act as a 'sink' that will probably prevent the delivery of sufficient amounts of the fusion protein to the tumour cells. In that case, additional modifications would be required, for example, adding a cleavage site for an intracellular enzyme that would remove the TAT-peptide from Apoptin after it has entered the cell, thereby preventing cell exit.

Triggering the immune system

Significantly, the tumour-specific actions of proteins such as Apoptin, MDA-7 or E4orf4 render them amenable to systemic therapy, which is likely the only solution to treat (micro)metastasis. The benefit of employing a general delivery system, instead of a less potent tumour-targeted system, stands or falls with the effect these proteins exert when they end up in normal cells. Nevertheless, the probability and extent of a specific immune response against these transgenes is an important question that currently remains unanswered. So far, expression of Apoptin *in vivo* has been carried out in human tumour cells xenografted in immune-compromised mice, and in the immune-competent transgenic mice a low level of Apoptin is continuously present after approximately 13 days of gestation, a situation that should result in immune-tolerance. At Berlex Biosciences, lung colonies of B16 mouse melanoma cells in the syngeneic, immune-competent Black 6 strain were treated with an i.v. injection of an Apoptin-plasmid complexed to liposomes. After three weeks, the experiment was terminated and a significant anti-tumour effect by Apoptin was observed compared to the anti-sense control (D. Henderson, unpublished data). In this study, there was no indication of an activation of the immune system, however, the time-span was relatively short and specific assays to detect an immune response were not used. Therefore, none of these studies are informative about the antigenic nature of the viral protein Apoptin. As treatment with Apoptin will likely comprise overexpression in immune-competent patients, the investigation of the effects of *Apoptin* overexpression in immune-competent mice is warranted. Evidently, the design of such studies can be improved when the delivery method that will be used for Apoptin has been established. In general, if normal cells overexpressing *Apoptin* become targets for cytotoxic T cells, the immune response elicited by Apoptin will comprise an unwanted side-effect of Apoptin treatment by causing damage to healthy tissues. In that case temporary inhibition of the immune system may prove beneficial. Blocking the costimulatory molecules CD40L, CD80 and CD86 effectively inhibits both humoral and cellular immune responses against adenoviral vectors [16], rendering this an attractive approach to test for Apoptin if future experiments demonstrate that immune repression is required.

Small molecular drugs based on Apoptin

The difficulties surrounding the delivery of Apoptin, either as a protein or gene, can be avoided by the use of a small molecular drug that mimics Apoptin's tumour-specific cell killing. Small molecular drugs are also more easily amenable to standardized and large-scale productions, both of which are prerequisites for clinical use. However, such a drug should be as effective and specific as Apoptin is; i.e., it should be based on the mechanism of Apoptin. Obviously, for the development of such a drug, elucidation of this mechanism is crucial. The data generated on Apoptin so far have resulted in our current model, which is based on tumour-specific activation of Apoptin rather than a difference in response of tumour versus normal cells to an otherwise identical Apoptin molecule.

Tumour-specific activation of Apoptin

The fact that Apoptin is active in all tumour cell types tested so far implies that Apoptin is activated by a cancer hallmark pathway, either by one of the hallmarks described in the Introduction, or by one that has yet to be identified. It could be that the breaching of checkpoints, the constitutive activation of a particular pathway, or the activation of several pathways simultaneously result in a molecular situation in tumour cells that never arises in normal cells, which may be responsible for the tumour-specific activation of Apoptin. On the other hand, it is also possible that the Apoptin-activating signal is occasionally present in normal cells, when a cancer hallmark pathway is employed for a physiological function, but that this activation is specifically prevented. This may be due to an unfavourable folding of Apoptin, to sequestration or shielding by an associating cellular protein or to enhanced degradation (figure 3).

The most convincing argument that differential activation of Apoptin is indeed responsible for its specific induction of apoptosis is the finding that the mutant that mimics phosphorylated Apoptin is able to trigger cell death in normal cells. Apparently, phosphorylation not only allows Apoptin to enter the nucleus but also to find its appropriate target there, since targeting Apoptin to the nucleus of normal cells does not elicit apoptosis.

Significantly, the kinase that phosphorylates Apoptin may be a crucial link in a hallmark cascade, and a small molecular drug designed to specifically inhibit this kinase could thus disrupt the tumour-essential pathway and subsequently impair malignant growth. Such inhibition does not automatically result in an apoptotic phenotype, as it depends on the characteristic endowed by the hallmark pathway. If the trait, for instance, would be forced progression through the cell cycle, the result of treatment with the Apoptin kinase-specific inhibitor could be growth arrest or perhaps differentiation. Depending on the localization of the tumour nodule(s), this therapy could still be effective in 'curing' cancer, as long as the remaining (arrested) cell mass did not hinder organ functions. However, an important consideration for an Apoptin kinase inhibitor is the possibility that activation of the Apoptin kinase is a side-effect of the cascade responsible for the tumour trait, e.g. when a kinase upstream of the Apoptin kinase activates a substrate crucial for the hallmark and the kinase for Apoptin in parallel (figure 3). In this case, activation of the Apoptin kinase would be a sensor for an active cancer hallmark pathway, but disrupting its activity will not lead to disruption of the hallmark pathway and therefore not be effective as an anticancer agent.

Regardless of whether the Apoptin kinase is essential for malignancy or not, preliminary experiments suggest that Apoptin kinase activity may indeed have diagnostic value. In tissue sections from patient material, the Apoptin kinase was remarkably more active in the tumour tissue than in surrounding normal tissue [14]. However, there was significant variation in the level of activity between tumours of different patients. Lower activity seemed to correlate with a lower percentage of actual tumour cells in the tested section; however, more observations are required to prove that upregulation of the Apoptin kinase indeed occurs in every tumour *in vivo*. It could also be that Apoptin is active in all tumour cells because it combines two death domains, so that

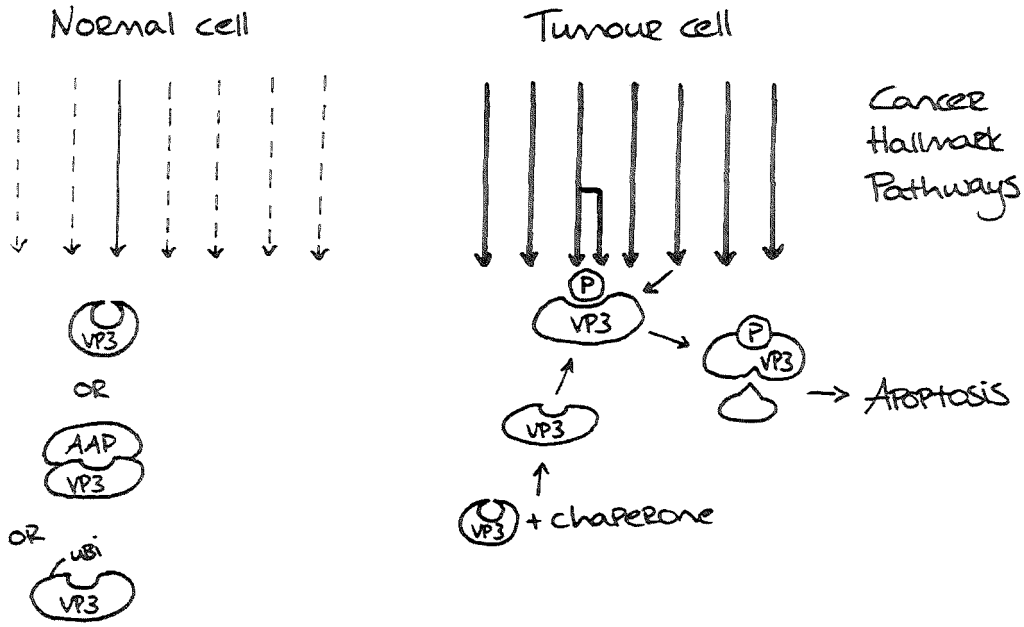

Figure 3. Model for the tumour-specific activation of Apoptin

For an in-depth discussion, see text. Briefly, Apoptin is phosphorylated specifically in tumour cells (right hand side of the model), which results in its activation (triangular indent), and subsequent activation of its target (extruding triangle), with the end result of apoptosis. Phosphorylation of Apoptin could be caused by a kinase that is active as part of a cancer hallmark pathway (chapter 1). It could also be that a protein in the cancer hallmark pathway activates the Apoptin kinase in parallel (forked pathway). Possibly, Apoptin responds to two cancer hallmark pathways simultaneously, for instance, if a conformational change induced by heat-shock proteins (potential cancer hallmark) is required to allow phosphorylation. Alternatively, a distinct pathway may activate the N-terminal death domain of Apoptin. In normal cells (left hand side of the model), the cancer hallmark pathway that activates Apoptin may never occur (e.g. breaching of a check point), or only rarely (e.g. heat shock upregulation following stress), or may not be constitutively overactive (as it would be in a tumour cell, e.g. overexpression of Cyclin D1 or mutated Ras). Furthermore, Apoptin may be improperly folded or the activation site may be shielded by an associating protein (AAP) or a normal-cell specific modification. In addition, Apoptin may be unstable in normal cells.

when one pathway is not active, e.g. the kinase, the other part, e.g. the pathway that activates the N-terminus, can still kill the cell. In that case, the kinase-inhibitor may not be effective in the tumours of patients with low kinase activity. Conversely, normal cells may crucially depend on small amounts of the Apoptin kinase and inhibiting it with a small molecular drug could be toxic.

In conclusion, it is important to investigate all possibilities and pitfalls for a therapy based on the inhibition of the Apoptin kinase, as such studies may facilitate the identification of tumour types that are less or more suitable for such treatment and thereby ensure the design of a clinical trial that will be most potent in validating (or falsifying) the Apoptin kinase inhibitor for

cancer therapy. Nevertheless, the discovery of the tumour-selective phosphorylation of Apoptin has yielded important proof of the existence of a tumour-selective mechanism and represents the best chance of designing an Apoptin-based molecular drug generated by the Apoptin research so far.

Normal-specific inhibition of Apoptin

Our data support a model where the difference in sensitivity to Apoptin between tumour and normal cells arises from the fact that Apoptin is specifically activated in tumour cells, whereas this activational change does not occur in normal cells (figure 3). A possible explanation for the strict tumour specificity of Apoptin may be due to the fact that it is dependent on two conditions that (almost) never occur at the same time in normal cells. In light of this hypothesis, upregulated heat shock proteins, which could be a cancer hallmark (see chapter 1), might activate Apoptin by creating a conformational change that allows binding to another cellular protein, resulting in phosphorylation and subsequent nuclear localisation. Electron microscopy shows that Apoptin is present in the ER of tumour cells prior to its nuclear transport, even though the Apoptin sequence lacks an ER-retention signal. In fact, one of the cellular binding partners of Apoptin identified in the yeast-two-hybrid screen of a transformed B-cell library was an ER-localised chaperoning protein called BiP. The possibility that Apoptin is activated by a stress-induced upregulation of chaperones is in keeping with anecdotal observations that stress may compromise normal-cell resistance to Apoptin. To investigate whether tumour-specific upregulation of heat-shock proteins is a determining factor for activation of Apoptin, normal cells expressing Apoptin could be subjected to heat to examine whether Apoptin is then also capable of inducing apoptosis in these normal cells. A negative result may indicate that Apoptin is regulated by multiple tumour-specific activities, and needs, for instance, phosphorylation after its proper folding before it can induce apoptosis. Downregulation of HSPs in tumour cells by antisense methods [17] could have answered the question whether heat-shock proteins are required for Apoptin-induced apoptosis, if this approach did not already induced cell death on its own.

Alternatively, the differential activity of Apoptin could be due to a phosphatase that is active in normal cells but specifically downregulated in tumour cells. Because the *in vitro* kinase assays that demonstrate phosphorylation of recombinant Apoptin by cell lysates from tumour cells but not from normal cells are performed in the presence of a cocktail of diverse phosphatase inhibitors, this seems unlikely [14]. Instead, phosphorylation may be prevented by association with a cellular protein specifically in normal cells which may or may not be determined by a normal cell-specific conformation of Apoptin. The biochemical studies demonstrating a shielded Apoptin-epitope in normal cells suggest that Apoptin may indeed be associated to a distinct cellular protein or differently folded than it is in tumour cells. Another putative inhibitory condition may be caused by covalent binding of ubiquitin moieties. Addition of one or more ubiquitin molecules to a protein can have a variety of effects, including conformational change, prevention

of phosphorylation, and targeting to lysosomes or proteasomes. Ubiquitylation of Apoptin occurs both in tumour and normal cells (chapter 5), indicating that an E3 ligase exists that can recognise Apoptin. Furthermore, studies demonstrating the disappearance of Apoptin recombinant protein in normal cells, as well as the stabilisation of Apoptin with proteasome inhibitors in *Apoptin*-transgenic cells, suggest that Apoptin may be unstable in normal cells. Therefore, ubiquitylation of Apoptin may have different effects on its stability in tumour versus normal cells. Whether the half-life of Apoptin is indeed significantly reduced in normal cells, comprising an additional safety feature for therapies with Apoptin, could be determined in pulse-chase assays.

Other drug targets

If cellular proteins apart from the kinase play a crucial role in the activation of Apoptin in tumour cells, they represent potential tumour-specific drug targets. The yeast-two hybrid screen has yielded twelve potential candidates for Apoptin-binding proteins in transformed cells [18]. However, the significance of these interactions for the activity of Apoptin must still be determined. A new technique has been developed that can facilitate the validation of these binding partners with regard to their role in Apoptin-induced apoptosis. By introducing small interfering RNAs, the expression of all candidates can be knocked down [19], and the study of Apoptin-associating proteins can then be limited to those that abrogate Apoptin-induced apoptosis when their expression is sufficiently reduced. Subsequent studies should categorise these essential Apoptin-binding partners as activators of Apoptin, which are likely to be tumour-specific, versus its downstream effectors, which are probably present in both tumour and normal cells, as the phosphorylation-mimic is capable of inducing apoptosis in normal cells. Significantly, none of the Apoptin-associating proteins had a kinase domain or resembled a known kinase regulatory subunit such as a cyclin. This could be because only a subset of mammalian proteins is screened, namely those present in the library. Moreover, transient associations are not detectable in a yeast-two-hybrid assay and it is also possible that a crucial Apoptin-associating protein must be modified by mammalian cell components before it can functionally interact. An additional screen in a mammalian system could help identify the Apoptin kinase, or at least aid the construction of a model for Apoptin activity by confirming some of the proteins isolated with the yeast-two-hybrid screen and identifying novel, may be more relevant, cellular-interacting proteins. The mammalian protein-protein interaction trap (MAPPIT)-method constitutes an attractive approach [20], provided that Apoptin does not induce apoptosis in tumour cells when it is tethered to the cell membrane.

If the upstream kinase cascade proves to be the major determinant of Apoptin's tumour-specificity, the situation may be fortunate, as kinases have proved to be much easier to inhibit than for instance protein-protein interactions. The ATP-pockets of kinases are 'druggable', i.e. represent an amenable target for small molecular inhibitors. Molecular studies of cancer have yielded a plethora of tumour targets, but only a subset of these is pharmaceutically tractable.

Receptor-ligand interactions, such as the receptor for EGF, and kinase activities, such as Bcr-abl, are good targets for drug development, as demonstrated by the success of therapeutic antibody Iressa and the small molecular inhibitor Gleevec, respectively. There is strong evidence that disruption of Myc/Max dimerization or Ras/Raf binding would inhibit the function of pathways essential to certain tumour cells. However, although inhibitory peptides were developed, their conversion into small molecular drugs with suitable pharmaceutical properties has failed [21]. Novel techniques such as high-throughput screens of peptidomimetic libraries may greatly increase the potential of this approach, but *in vivo* success has not yet been achieved [22]. Therefore, while awaiting validation of the Apoptin kinase as a tumour essential effector and considering the potential lack of additional druggable targets, small molecular drugs need to be developed side-by-side with gene and protein therapy, in the hope that at least one of these approaches will produce a successful anti-cancer agent based on Apoptin.

Future of tumour-specific therapies

The research described in this thesis is a first exploration of the potential of Apoptin as an anticancer agent. I showed that when a substantial percentage of a tumour *in vivo* is infected with an adenoviral vector expressing *Apoptin*, the tumour mass disappears. Importantly, no adverse effects were detected in normal tissues, not even after intravenous injection of healthy rats. The generation of transgenic mice provided further evidence for the lack of harmful activity of Apoptin in normal cells, as well as new research opportunities. Together, these findings support the potential of Apoptin for cancer therapy. At the same time, they revealed the inadequacy of replication-deficient vectors for this purpose. Development of alternative gene or protein delivery techniques for Apoptin is warranted, not only because of the clear anti-tumour effects achieved *in vivo* with the adenoviral transduction of Apoptin, but also because recent *in vitro* data further corroborate the potential of Apoptin as an anti-cancer agent. For instance, it has become clear that Apoptin acts downstream of most apoptosis regulators, rendering Apoptin a potent inducer of apoptosis regardless of the individual evasive mutations acquired by tumours (Danen, chapter 1 and 4). This could mean that Apoptin may be active in tumour cells that are resistant to certain other cancer therapies. In addition, Apoptin was shown not to harm primary human cell types that are highly sensitive to classical and certain novel cancer therapeutics, such as hepatocytes and $CD34^+$ stem cells [12] and Zhang, personal communication). If the same holds true *in vivo*, Apoptin's selectivity would provide a therapeutic window that exceeds existing ones. Equally exciting is the observation that Apoptin is tumour-specifically modified, providing the first prospect for small molecular drug intervention. A small molecular drug approach would not be hindered by delivery problems, but it remains to be determined whether such an approach would lead to tumour-specific cell death or restraint, and whether the target is indeed 'druggable'. Since the tumour-selective properties of Apoptin have already been demonstrated, it would be advisable to continue the development of delivery methods for Apoptin in parallel.

As discussed, it will be important to gain further knowledge about the mechanism of Apoptin activation and subsequent effects. Because Apoptin is active in all tumour cells tested to date, it is likely that it detects a tumour essential pathway, probably quite downstream. Systematic testing of all known cancer hallmark pathways for their activation of Apoptin should provide important information for the development of the Apoptin kinase-inhibitor, identification of new drug targets as well as for the design of combination therapies. If Apoptin is activated by one or more of the known cancer hallmark pathways, it may be that an inhibitor of this pathway already exists. In this case, the Apoptin research may induce the testing of this inhibitor as an anticancer agent. It may also be that inhibitors upstream in this pathway are not effective in all tumour cells, or cause side effects due to their involvement in other pathways, in which case a new inhibitor based on Apoptin, for instance inhibiting Apoptin kinase, may provide a more potent or selective alternative to target this particular pathway for cancer therapy. If Apoptin responds to a presently unknown cancer hallmark pathway, this could yield new targets for cancer treatment, as well as new insight in the process of carcinogenesis. Arguably, Glivec, Tarceva and Iressa were developed in parallel with the elucidation of the relevant molecular defects. The lack of detailed understanding did not slow down the development of clinical candidates, and likewise the new understanding of molecular pathways involved had only a limited impact in accelerating the development process [21], suggesting that the development of Apoptin applications may not be crucially dependent on elucidation of its mechanism.

Nevertheless, it will be important to know what the mechanism of Apoptin is, as it will allow for rational combination with other cancer therapeutics. It currently seems clear that tumour cells must acquire a limited set of essential traits, as specified by the cancer hallmarks, and that this requirement allows the design of tumour-specific treatment modalities. However, this fact does not change the reality that each tumour has acquired a unique combination of mutations leading to the malignant phenotype. Considering the possibility that genetic instability in tumours can lead to therapy-resistant clones, the most effective cancer therapy will most likely be based on multiple agents targeting several tumour essential pathways simultaneously. Notwithstanding the recent successes of the molecular therapy of cancer, it is apparent that not all of the approaches are performing as well as anticipated. There is clearly a learning curve with respect to the best ways to use these new agents, just as has been the case in the development of traditional cytotoxic drugs. A number of toxicities have been encountered, some of which are mechanism-based (EGF receptor inhibitors, FTIs, MMPIs) and some of which are caused by the chemical structure of drug in a manner that is unrelated to its mechanism of action (phosphothioate antisense oligonucleotides) [21]. Furthermore, several patients have in fact developed resistance to Gleevec, either by amplification of BCR-ABL or by selection for mutations affecting the ATP-pocket, rendering a perturbation of drug binding that leaves ATP intact [23]. Fortunately, Gleevec-resistant clones can be eliminated either by a farnesyl transferase inhibitor or by the Hsp inhibitor 17-AAG (see introduction, and refs [24,25]), illustrating the power of targeting distinct tumour pathways simultaneously.

In the case of Apoptin, the effect of radiation or chemotherapy on tumour cells was shown to be significantly augmented by additional Apoptin treatment, both *in vitro* and *in vivo*. Specifically, when irradiation of mice bearing human prostate tumours was combined with the injection of a tumour-targeted liposome containing the *Apoptin* gene, the tumour growth was reduced an additional 6-fold compared to radiation alone (unpublished data, E. Chang). In addition, S. Olijslagers demonstrated that infection of breast or prostate cancer cells with AdApt.*Apoptin* significantly reduced the amount of taxanes required to kill these cells [26]. Importantly, when the mechanism of Apoptin is elucidated, an Apoptin-based treatment can be rationally combined with tumour-specific therapies that target a distinct pathway. Ideally, such a combination would prevent the occurrence of therapy-resistant clones because the chance of the emergence of a tumour cell that has acquired two mutations in two separate pathways simultaneously is much smaller than the chance of generating resistance to an agent targeting only one pathway. Hopefully, the combination of several tumour-specific agents will be so potent that the required doses will be lower, subsequently limiting toxicity.

Concluding remarks

Taken together, the effects of Apoptin *in vivo* merit further investigation, of both its activity in naturally-arising tumours and its potential toxicity in normal tissues. The generation of a new *Apoptin*-transgenic mouse with a ubiquitously and highly active promoter would expand the technical possibilities to examine the effects of Apoptin in normal cells and simultaneously describe the window of opportunity for delivery methods. Protein transduction delivery *via* the TAT-peptide or conditionally-replicating adenoviral vectors may constitute more effective methods for the delivery of the Apoptin gene and protein. In addition, continuous research into the mechanism of Apoptin is important for the prediction of potential toxicities, the development of small molecular drugs and the design of combination therapies.

Several decades ago, the application of chemo- and radiation therapy had a dramatic impact on the prospects of cancer patients. Since then, few advances have been made in cancer research that translated into actual clinical benefits. In recent years, however, the accumulating knowledge of the molecular biology of cancer has started to pay off. Eventually, after a scientific learning curve and feedback from clinical trials, molecularly based medicines should be able to afford more effective cancer therapies with diminished side effects. Exploring the remarkable tumour selectivity of Apoptin could make an important contribution to this field. Nevertheless, the outcome of numerous interesting experiments, some of which have been proposed in this discussion, will determine whether an Apoptin-based medicine will be among the few that meet the clinical criteria.

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Nederlandse samenvatting

Inleiding

Therapieën tegen kanker zijn tot nu toe gebaseerd op het feit dat tumorcellen over het algemeen sneller delen dan normale cellen. Zowel chemotherapie als bestraling veroorzaken zodanige schade in cellen dat zij ofwel onmiddellijk doodgaan, of dat doen op het moment dat zij proberen te delen voordat de schade hersteld is. Reparatie van schade gaat over het algemeen gepaard met een tijdelijke, en soms permanente, rem op de celdeling. In tumorcellen is deze inhibitie van de celdeling vaak uitgeschakeld, tumorcellen blijven zich immers per definitie vermeerderen. Dit leidt ertoe dat tumorcellen eerder doodgaan door chemotherapie of bestraling dan normale cellen die in sommige gevallen de schade kunnen herstellen. Dit neemt niet weg dat de bijwerkingen van deze behandelingen aanzienlijk zijn, doordat organen waarin veel celdelingen plaatsvinden ernstige schade oplopen. Weefsels die in hoog tempo cellen vervangen zijn bijvoorbeeld de darmen of het beenmerg dat nieuwe bloedcellen aanmaakt. De gevoeligheid van gezonde weefsels voor kankertherapieën bepaald in hoge mate het succes van de behandelingen; in veel gevallen zou een hogere dosis bestraling of chemo resterende tumorcellen kunnen doden, ware het niet dat de patiënt zo'n behandeling niet zou overleven. Nieuwe behandelingen voor kanker zullen dus bij voorkeur gericht moeten zijn tegen eigenschappen die specifiek zijn voor de tumorcellen, en die niet of nauwelijks voorkomen in gezonde cellen.

Kankertherapie gericht op tumor-specifieke eigenschappen, in plaats van op celdeling in het algemeen

Onderzoek naar de moleculaire verschillen tussen tumor- en normale cellen (wat maakt een tumorcel een tumorcel?) heeft laten zien dat tumorcellen niet slechts normale cellen zijn die teveel delen. Er blijken allerlei beschermingsmechanismen in normale cellen te bestaan die allemaal geïnactiveerd zijn in tumorcellen. Die inactivatie gebeurd niet 'bewust', maar is een gevolg van het elke keer opnieuw kopiëren van het genetische materiaal (de genen, opgebouwd uit DNA) op het moment dat een cel deelt. Dit is een heel nauwkeurig proces, maar het is niet perfect en er treden dus af en toe fouten, of mutaties, op. Vaak hebben deze mutaties geen effect, of leiden tot celdood, maar in een enkel geval zorgen ze ervoor dat een beschermingsmechanisme tegen ongebreidelde celgroei niet meer werkt. Zo'n cel heeft de kans om uit te groeien, te vergelijken met de 'survival of the fittest' van individuen in de natuur, maar dan als een individuele cel in een lichaam. Als een dergelijke cel kan blijven delen, heeft het vervolgens een hogere kans op een nieuwe mutatie tijdens een volgende celdeling. Die mutatie kan een nieuwe eigenschap opleveren die bijdraagt aan de ontwikkeling tot tumorcel. Maar de kans dat dit een ongunstige eigenschap voor de tumorontwikkeling is, is vele malen groter. Dat

is een van de verklaringen voor het feit dat het vaak jaren duurt voordat mensen tumoren krijgen; rokers krijgen bijvoorbeeld pas na tien tot twintig jaar longkanker. Terug naar het begin van deze alinea: tumorcellen blijken dus tijdens een langdurig proces geselecteerd te worden op het vergaren van mutaties die leiden tot tumor-specifieke eigenschappen, zoals bijvoorbeeld de inactivatie van een rem op de celdeling, het voorkomen van een zelfmoordprogramma dat wordt aangeschakeld als een cel een gevaar voor het lichaam dreigt te worden, de ongevoeligheid voor signalen van buurcellen die een cel laten weten dat er genoeg gedeeld is, en nog een aantal andere.

Het ontdekken en onderzoeken van deze tumor-specifieke eigenschappen heeft ertoe geleid dat nieuwe kankertherapieën ontwikkeld zijn die gericht zijn tegen een van deze eigenschappen, en dus minder bijwerkingen zouden moeten hebben en (daardoor) effectiever kunnen zijn dan de bestaande behandelingen omdat normale cellen niet aangetast worden. Veel van deze nieuwe therapieën zijn nog in de ontwikkelingsfase, maar een klein aantal zijn onlangs goedgekeurd als officieel medicijn, zoals bijvoorbeeld Glivec tegen leukemie of Herceptin tegen borstkanker.

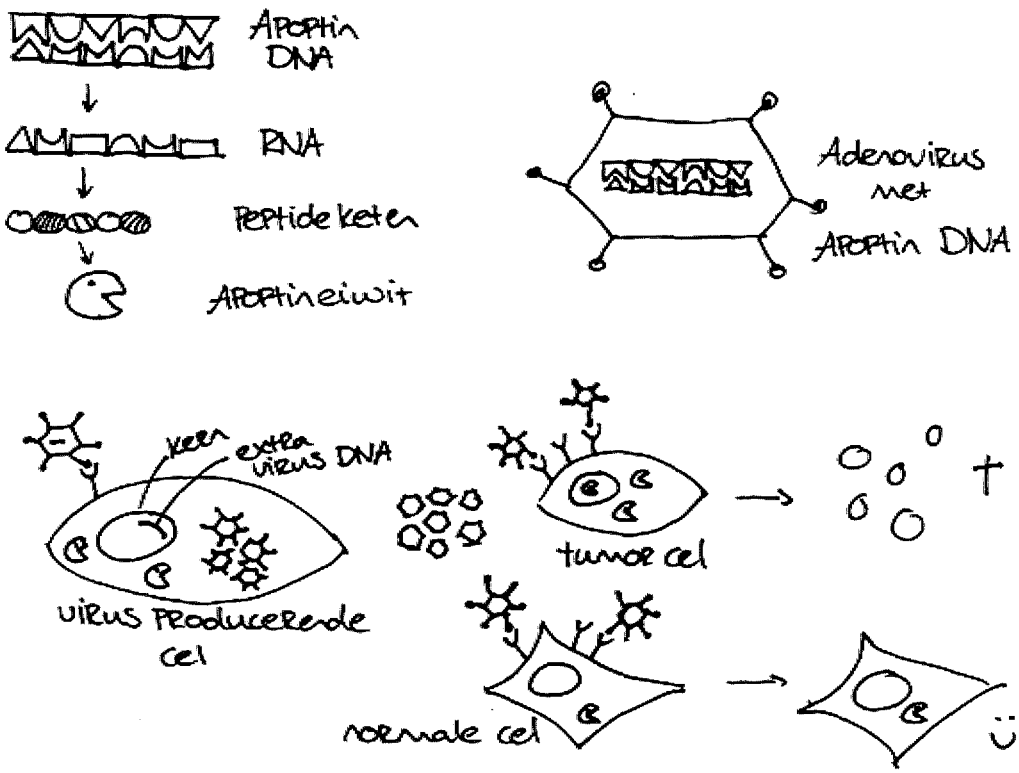
Onderzoek naar mogelijke tumor-specifieke therapieën gebeurt op basis van inmiddels bekende tumor-eigenschappen, het ontdekken van nieuwe tumor-eigenschappen, of op basis van moleculen waarvan bekend is dat ze tumor-specifiek zijn, maar waarvan de bijbehorende tumor-eigenschap onbekend is. Het onderzoek dat ik beschrijf in dit proefschrift is gebaseerd op de laatst genoemde mogelijkheid. Het eiwit Apoptin veroorzaakt celdood in tumorcellen en niet in normale cellen, maar we weten nog niet waarom.

Dit proefschrift

Apoptin is een eiwit, en eiwitten zijn de producten van de genen die in een cel zitten en de uiteindelijke functies uitoefenen die nodig zijn voor een cel. Nu komt het gen dat Apoptin maakt niet voor in cellen, het is afkomstig van een virus. Virussen bevatten slechts genen met een kapsel eromheen, en hebben verder geen mogelijkheid om zelfstandig van deze genen de functionele producten, of eiwitten, te maken. Op het moment dat een virus een cel binnenkomt ('infecteert') gebruikt het de eiwitten van de cel om van zijn eigen genen viruseiwitten te laten maken. Dit leidt uiteindelijk tot vele nieuwe virusdeeltjes die de cel verlaten en weer nieuwe cellen kunnen infecteren. Het gen voor Apoptin komt van een virus en zal dus waarschijnlijk bijdragen aan het proces van virusproduktie, maar ook daar is weinig over bekend. Wat wel bekend is, is dat als het gen voor Apoptin in tumorcellen van een mens (of muis) wordt gebracht, en dus het eiwit Apoptin wordt gemaakt ('tot expressie gebracht'), deze cellen na een aantal dagen hun zelfmoordprogramma aanschakelen en vervolgens doodgaan. Wanneer dit gen in normale cellen tot expressie wordt gebracht, lijken deze cellen er totaal geen last van te hebben. Het lijkt niet uit te maken wat voor celtype het is, Apoptin heeft bijvoorbeeld geen effect in huidcellen, bloedcellen of levercellen, maar wel in de tumorcellen die uit deze weefsels kunnen ontstaan.

Deze proeven zijn allemaal gedaan met gekweekte cellen, dus cellen die uit het lichaam zijn gehaald en in schaaltjes in leven worden gehouden. Het Apoptin gen is in deze cellen gebracht met een techniek die alleen op cellen in schaaltjes werkt en niet erg efficiënt is; slechts een deel van de cellen neemt het gen op en brengt Apoptin tot expressie. Toen ik begon aan mijn AIO-periode waren wij benieuwd of Apoptin ook echt te gebruiken zou zijn als kankertherapie. Om dat te kunnen testen, moest er een andere techniek gebruikt worden, waarmee Apoptin efficiënt in cellen gebracht kon worden, niet alleen in een schaaltje maar ook in een echte tumor in een levend organisme (uiteindelijk misschien ooit in de mens, maar in eerste instantie in de muis). Aangezien virussen geëvolueerd zijn om hun genen zo efficiënt mogelijk in cellen te krijgen zodat er nieuwe virusdeeltjes gemaakt kunnen worden (de virussen die dat niet kunnen verdwijnen vanzelf), heb ik het gen voor Apoptin in een virus gezet dat heel efficiënt een groot aantal celtypes kan infecteren en dat vaak voor dit soort studies wordt gebruikt. Dit virus is gebaseerd op een humaan verkoudsheidvirus, het Adenovirus genaamd, maar er zijn een aantal genen uitgehaald waardoor het geen nieuwe virusdeeltjes meer kan vormen als het in een cel komt, het brengt slechts het gen dat de onderzoeker erin gezet heeft tot expressie. Op deze manier is de enige overgebleven functie van het virus het efficiënt naar binnenbrengen van het gen, als bezorger of 'carrier'. Om nu toch voldoende virusdeeltjes met het Apoptin gen te krijgen om een tumor mee te infecteren heb ik gebruik gemaakt van cellen die zo geconstrueerd zijn dat ze de ontbrekende virusgenen in hun DNA hebben, en dus de eiwitten maken die nodig zijn voor virusproduktie. Deze virusproducerende cellen zijn tumorcellen, en de eerste belangrijke test was dan ook of deze cellen wel Apoptin virus konden maken, of dat zij voor die tijd al dood zouden gaan door Apoptin. Gelukkig hebben de cellen maar twee dagen nodig om virus te maken, en gaan ze pas na een dag of vier dood door Apoptin, wat betekent dat de techniek die ik gekozen had bruikbaar was (hoofdstuk 2). Het volgende belangrijke punt was of Apoptin nog wel tumor-specifiek zou zijn als het op deze nieuwe manier in cellen werd gebracht. Ook dat bleek het geval, infectie van menselijke levertumorcellen liet zien dat Apoptin deze cellen doodde, terwijl het geen effect had op normale levercellen uit een rat (figuur 1).

Vervolgens konden we voor het eerst gaan kijken wat de gevolgen waren van expressie van Apoptin in een organisme. Hiervoor hebben we dezelfde menselijke levertumorcellen onderhuids gespoten bij naakte muizen, een muizenstam (zonder vacht) die geen goed afweersysteem heeft en menselijk weefsel daardoor niet afstoot. Deze cellen groeien uit tot een echte tumor, met ondersteunend weefsel, verschillende compartimenten en een bloedvoorziening. De tumoren hebben we na drie weken geïnjecteerd met het Apoptin virus, en ook met een controle virus of alleen een zout-oplossing (hoofdstuk 3). Uit deze experimenten bleek dat in 30% van de tumoren die met Apoptin-virus behandeld waren, deze geheel verdwenen, terwijl dat niet gebeurde met de controle behandelingen. Een ander deel van de Apoptin-behandelde tumoren groeide minder hard of nam in omvang af, maar bleef wel aanwezig. En op de overige tumoren had de Apoptin-behandeling zelfs nauwelijks effect. Toen we doorsnedes van de tumoren bekeken bleek hoe dat kwam, namelijk door de verschillende compartimenten in de tumoren waardoor het virus niet overal terecht was gekomen. Het effect van de therapie hing dus af van de manier waarop de



Figuur 1. Gentherapie met Apoptin

De specifieke volgorde van DNA bouwstenen (een gen) bevat de informatie voor een cel om verschillende peptides zodanig aan elkaar te koppelen dat een eiwit gemaakt wordt, in dit geval Apoptin. Dit gaat via de intermediar RNA, die de informatie vanuit de kern (waar de informatie ligt opgeslagen) naar het cytoplasma (waar eiwitten worden geproduceert) brengt. Het gen voor Apoptin is in de plaats gezet van genen van het adenovirus, waardoor dit virus zich in een willekeurige cel niet meer kan vermenigvuldigen. Dit kan nog wel in specifieke virus-producerende cellen, waar onderzoekers de ontbrekende virusgenen in het cellulaire DNA hebben geplaatst. Na infectie met het Apoptin-virus maken deze cellen een grote hoeveelheid nieuwe virusdeeltjes met het Apoptin gen (en maken ook Apoptin eiwit). Deze virussen kunnen vervolgens gebruikt worden om het Apoptin-gen efficiënt in normale en tumorcellen te brengen. In deze cellen, bijvoorbeeld in een weefselkweekschaaltje of in een muis, ontstaan dus geen nieuwe virusdeeltjes meer, er wordt slechts Apoptin eiwit geproduceerd. Als in tumorcellen het eiwit Apoptin wordt gemaakt, bevindt dit eiwit zich zowel in het cytoplasma als in de kern van de cel en wordt een al aanwezig zelfmoordprogramma van de cel, apoptose genaamd, aangezet. In normale cellen daarentegen blijft het geproduceerde Apoptin eiwit uitsluitend in het cytoplasma en lijken de cellen er geen last van te hebben.

injecties plaats hadden gevonden en hoeveel tumorcellen ook daadwerkelijk het Apoptin-virus hadden opgenomen. De belangrijkste conclusie uit deze experimenten is dat als Apoptin in tumorcellen terecht komt het in staat is deze cellen te doden, maar dat deze virus-carrier niet efficiënt genoeg is om Apoptin in alle cellen van een tumor tot expressie te brengen. Op dit moment wordt er gezocht naar een andere manier om Apoptin in tumorcellen te brengen. Een alternatief is om te onderzoeken waarom Apoptin actief wordt in tumorcellen, maar niet in normale cellen. Als dat proces bekend is, kan geprobeerd worden een klein molecuul te maken dat net als chemotherapie door alle cellen opgenomen wordt. Dit molecuul zou dan wel moeten reageren op dezelfde tumor-eigenschap waar Apoptin op reageert, en ook tot gevolg hebben dat de tumorcel specifiek doodgaat. Ook hier wordt aan gewerkt.

Om nog meer inzicht te krijgen in de effecten van Apoptin in een levend organisme, zonder afhankelijk te zijn van een transductie-techniek, hebben we een Apoptin-transgene muis gemaakt (hoofdstuk 5). Deze muis heeft al zijn eigen genen, met als extra het gen voor Apoptin. Omdat embryonale cellen in principe een aantal 'tumor-achtige' eigenschappen hebben, waren we niet zeker of deze muizen wel geboren konden worden. Voor de zekerheid hebben we gekozen een stukje DNA aan het Apoptin-gen te zetten dat ervoor zorgt dat Apoptin pas laat in de embryogenese tot expressie komt. De Apoptin-muizen werden geboren en er waren geen tekenen van afwijkingen, wat suggereert dat Apoptin geen nadelig effect had op de (normale) cellen van deze muizen. Het stukje DNA dat de relatief late Apoptin expressie bepaalde, heeft ook invloed op welke celtypen Apoptin uiteindelijk tot expressie brengen, en het bleek dat de Apoptin-transgene muizen het Apoptin eiwit met name tot expressie brachten in cellen van het afweersysteem. Deze cellen zijn onderdeel van het bloed, en hebben in de mens erg te leiden van chemotherapie en bestraling. Als normale afweercellen niet gevoelig blijken te zijn voor Apoptin dan zou dit een belangrijk argument vormen om Apoptin verder te testen als anti-kanker middel. En als de muizen tumoren zouden krijgen afkomstig van deze afweercellen ('lymphomen') en het aanwezige Apoptin zou dat kunnen voorkomen, dan zou dat voor de eerste keer zijn dat Apoptin geen tumorcellen doodt die al eerder gekweekt zijn in een schaaltje, maar tumoren die ter plekke ontstaan. Uit mijn proeven met deze muizen blijkt dat de afweercellen inderdaad geen last hebben van de Apoptin expressie. Maar tegelijkertijd toonde ik aan dat het Apoptin eiwit in deze cellen niet erg stabiel is. Nu zijn er aanwijzingen dat Apoptin misschien stabieler wordt als een cel een tumorcel wordt, dus het zou kunnen dat dit een eigenschap is die bij Apoptin hoort. Maar het kan ook dat het Apoptin gen ongelukkig terecht is gekomen in het DNA van de transgene muis of dat Apoptin om de een of andere reden niet functioneel is. Voordat ik de conclusie kan trekken dat Apoptin niet schadelijk is voor afweercellen, moet er eerst aangetoond worden, dat als deze cellen tumorcellen worden, het Apoptin eiwit in deze cellen wel degelijk in staat is ze te doden. Deze proeven zijn helaas nog niet afgerond.

Als er uiteindelijk een manier is gevonden om een tumor-specifieke therapie te ontwikkelen op basis van Apoptin, door het gen of eiwit efficiënt in tumorcellen te brengen, of door een klein molecuul te maken dat werkt volgens hetzelfde mechanisme, en de dierproeven laten vervolgens

gunstige resultaten zien, dan zou zo'n therapie getest kunnen worden op mensen. Bijvoorbeeld bij patiënten met tumoren van de galgang (cholangiocarcinoma). Op het moment is er geen behandeling mogelijk van galgangtumoren, omdat ze vaak vertakt zitten in de lever en dus moeilijk weg te snijden zijn, en omdat ze ongevoelig zijn voor bestraling en chemotherapie. Vooruitlopend op de ontwikkeling van een Apoptin-therapie, heb ik bekeken of deze resistente tumorcellen dan misschien wel gevoelig waren voor Apoptin. Uit verschillende proeven bleek dat Apoptin zeer effectief tumorcellen afkomstig van drie verschillende patiënten doodde (hoofdstuk 4), wat een additioneel argument vormt voor het verder ontwikkelen van een Apoptin-therapie.

Conclusie

In het kort laat dit proefschrift zien dat het technisch haalbaar is een Apoptin-virus te maken, dat Apoptin in deze context tumor-specifiek blijft en ook daadwerkelijk muizen van geimplanteerde tumoren kan genezen. Tegelijkertijd laat het ook zien dat er een efficiëntere techniek nodig is om Apoptin in alle cellen van een tumor te krijgen. Verder zijn Apoptin-transgene muizen levensvatbaar en lijken de afweercellen van deze muizen geen last te hebben van Apoptin expressie, alhoewel dit verder moet worden uitgezocht. Uiteindelijk zouden bijvoorbeeld galgangtumoren in aanmerking kunnen komen voor een therapie op basis van Apoptin, maar dit hangt af van de technische mogelijkheden om zo'n therapie daadwerkelijk te ontwikkelen.

Dankwoord

Een promotieonderzoek in de moleculaire en medische biologie is niet alleen interessant en spannend, het betekent ook lange dagen, dicht op elkaar met een kleine groep mensen, groepsleiders die veel van onderzoek weten maar weinig van management, en veel mislukte proeven. Dat dit boekje er uiteindelijk toch is gekomen, is mede dankzij enthousiaste en bevriende collega's en de relativerende aanwezigheid en steun van mensen buiten 'de wetenschap'. Deze pagina's zijn speciaal om aan een groot publiek te laten weten welke kleurrijke personen het allemaal de moeite waard hebben gemaakt ...

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Daarna werd Leadd te groot en verhuisden we naar de Annex. Gelukkig werd ons kleine groepje snel uitgebreid met Frederique (met een groot hart), Jenny (thanks for reading all my stuff!), Patrick (computer-goeroe en Star trek bron), Anne-Marijke (gezellig aanwezig) en natuurlijk Maud en Klaas. Maud, zoals wij samen proeven kunnen doen zijn er maar weinigen denk ik. Alhoewel het beeld van ons samen met de brug van een RNA blot in onze handen me onveranderelijk aan het lachen krijgt, hebben we erg veel voor elkaar gekregen, zowel met vier handen als ieder apart. Ik heb bewondering voor je stoere levensinstelling en inlevingsvermogen en genoten van ons discussies, over wetenschap, het nieuws, en natuurlijk de man/vrouw verhouding waarbij Klaas onmisbaar was. Zonder verschillend standpunt geen discussie, en Klaas, gezellige bench-buur, was een gewillig slachtoffer of aanstichter al naar gelang het onderwerp. En altijd in voor een feestje, die we dan ook regelmatig hebben gehad.

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Lieve vrienden en familie, mijn wereld is mooier met jullie,

Alexandra

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