APOPTOSIS AND ANNEXIN V

APOPTOSE EN ANNEXINE V

PROEFSCHRIFT

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PROF. DR P.W.C. AKKERMANS M.A.

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Promotor:	Prof. dr H. Galjaard
Overige leden:	Prof. dr R. Benner Prof. dr B. A. Oostra Dr C. P. M. Reutelingsperger
Co-promotor:	Dr Chr. Vermeij-Keers

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Aan Annemarie en Sanne
Aan mijn ouders en schoonmoeder
In herinnering aan mijn schoonvader

Contents

General introduction

Degenerating cells, following specific spatiotemporal patterns were observed in developing specimens as early as the mid 19th century (reviewed in: Clarke and Clarke, 1996)[1]. After having been out of focus for almost a century, this "shrinkage necrosis" regained interest by developmental biologists after Glücksmann's review (Glücksmann, 1951), showing that this kind of cell death with its peculiar cell morphology was intimately linked to the normal development of both invertebrate and vertebrate species. It is, however, to the credit of Kerr, Wyllie and Currie that the concept of a physiological cell death reached a wide scientific community (Kerr *et al.*, 1972). These authors revealed that degenerating cells that showed cellular and nuclear pyknosis, and which were rapidly phagocytosed, were an integral part of both developmental processes and tissue homeostasis of the adult; they dubbed this process of cell death with the term apoptosis. Ultrastructural investigations revealed that during the apoptotic process organelles stay largely intact, but are frequently more densely packed and clustered according to organelle type. Some changes of organelles which may be observed in apoptotic cells include the dilation of the endoplasmatic reticulum which eventually may fuse with the plasma membrane, and the presence of crystalline areas of ribosomes (Wyllie *et al.*, 1980; Duvall *et al.*, 1986). By these morphological characteristics, apoptosis markedly differs from the type of cell death which is frequently observed in tissues that have been exposed to high levels of toxic agents or strong physical stimuli such as intense heat, cold or ischemia, *i.e.* necrosis, or accidental cell death. Necrotic cells, are characterized by an increase in cell-volume, swelling of organelles and loss of plasma membrane integrity before the cells are cleared by phagocytes (Majno *et al.*, 1995).

Apoptosis: physiological cell death

The first apoptotic cells that can be observed in developing mammalian specimens are probably those located centrally in the inner cell mass, forming the amniotic cavity out of an initially solid embryonic structure (Coucouvanis *et al.*, 1995). Shortly thereafter, cells derived from all three germ layers, *i.e.* ectoderm, endoderm and mesoderm, die at specific locations, within discrete time windows (Glücksmann, 1951; Saunders Jr. *et al.*, 1966; Clarke *et al.*, 1996). An important physiological function for apoptosis during normal development is suggested by its apparent close spatiotemporal relationship with morphogenetic processes such as cavitation, fusion, and differential growth; some profound examples of these processes are the recanalization of the anal canal, fusion of the neural walls, and shaping of the neural tube into spinal cord and brain, respectively (Hoving *et al.*, 1990; Blaschke *et al.*, 1995; Nievelstein *et al.*, 1998). In addition, throughout life apoptosis appears to be related to maintaining tissue achitecture and homeostasis, by effecting the deletion of supernumerous or unwanted cells (Kerr *et al.*, 1972; Oppenheim *et al.*, 1977; Wyllie, 1992). Whereas these studies show that apoptosis is an integral part of normal physiology, clearest evidence for the importance of this process probably comes from data showing that an abnormal occurrence of apoptosis may leed to congenital malformations and disease.

[1] References of this chapter are to be found on pages 83 to 94 of this thesis

Apoptosis and congenital malformations

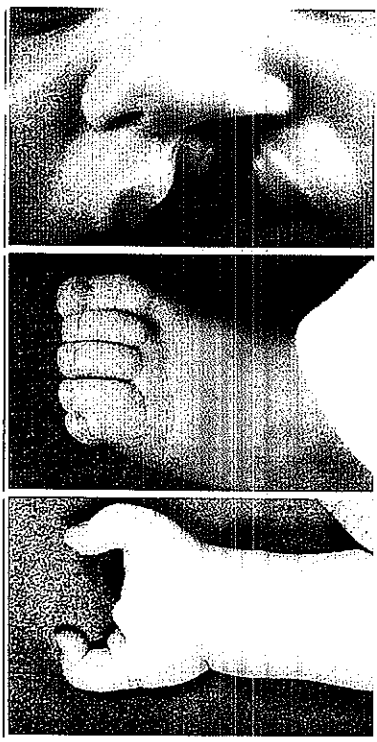

Figure 1: Examples of congenital malformations which may result from a shortage of apoptosis (top, cleft lip; middle, interdigital webbing [digits 4-5]) or by an excess of apoptosis (bottom, oligodactyly).

Deviations from the physiological presence or absence of developmental cell death may result in congenital malformations of both internal organs and external parts of the body. Examples of internal organs affected by a surplus of apoptosis are the reduced numbers of nephrones in mice defective for the cell death-inhibitory protein Bcl-2 (Nagata *et al.*, 1996) and in patients with polycystic kidney disease (Woo, 1995). Also nitrofen induced congenital diaphragmatic hernia seems to be related to an intensivation of cell death, involving a reduction of mesodermal cells adjacent to the transverse septum, which normally would populate the diaphragmatic anlage (Alles *et al.*, 1995).

The most commonly known apoptosis related congenital malformations show at the outer surface of the body (Figure 1). An example is the cleft lip which seems to be related to a decrease in cell death of the ectoderm covering the opposing and/or adhering maxillary and lateral- and medial-nasal swellings during fusion (Vermeij-Keers *et al.*, 1983). In general, the type of malformation reflects the time window in which the normally occurring apoptosis patterns were disturbed. This is probably best illustrated by the various congenital limb malformations that can develop after exposure to teratogens, or are caused by genetic aberrations. Embryologically, limbs are extensions of the body that grow from their distal ends, and in that respect can be considered as a time line with relatively early generated elements located proximally and late generated elements distally. This implies that overexpression of cell death from early to late stages of development can subsequently result in the absence of (a) complete limb(s) (Nomura *et al.*, 1996), radius agenesis or aplasia (Knudson and Kochar, 1981) and reduction anomalies of more distal elements such as fingers or phalanges (van der Zee *et al.*, 1996). In contrast, lack of apoptosis, may result in polydactyly (Knudson and Kochar, 1981; Bynum, 1991) and interdigital webbing (van der Hoeven *et al.*, 1994; Zakeri *et al.*, 1994). While many of these congenital malformations are compatible with life, it is clearly that they may cause both functional and psychosocial handicaps.

Apoptosis and disease

A most striking example of the pathological effects of an excess of cell death is the acute insult after vascular occlusion and subsequent ischemia of brain and heart tissue, leading to a center of necrotic neurons or cardiomyocytes, which is often bordered by a mixed population of apoptotic *and* necrotic cells (Gottlieb *et al.*, 1994). It has been suggested that apoptosis is not only linked with acute lesions of the heart, but cardiomyocyte apoptosis is also a frequently observed phenomenon in the decompensated heart, where it may contribute to the progress of disease (Olivetti *et al.*, 1997). Similarly, neurodegenerative disorders may be linked with a variety of other

factors than low oxygen. This is for instance indicated by studies of Lurcher mice. These mice suffer from ataxia, which has been shown to be caused by selective apoptosis of cerebellar Purkinje cells in the first few weeks after birth (Norman *et al.*, 1995). In addition, neurons have been observed to die by apoptosis in brains of adult mice with, for example, scrapie (Lucassen *et al.*, 1995).

In contrast to the previous examples which show that the normal physiology of some tissues is probably best served by limiting the number of apoptotic cells, other studies, in particular regarding the immune system and tumor biology, have indicated that the presence of apoptosis may be crucial to the maintaining of homeostasis. Namely, autoimmune diseases and inflammatory responses in normally immune privileged tissues like the eye and nervous system are probably caused by an incomplete deletion of potentially self reactive lymphocytes in the thymus and in peripheral tissues (Baixeras *et al.*, 1994; Griffith *et al.*, 1995; Gold *et al.*, 1997). But also the removal of potentially malignant cells is mediated by apoptosis (Kerr *et al.*, 1994). Among the many cancer related genes identified sofar, the most extensively studied is *p53*. This gene encodes a protein, also known as the guardian of the genome, which activates apoptosis of potentially malignant cells with damaged and inadequately repaired DNA (Lowe *et al.*, 1993). Interestingly, by comparing litter sizes and incidence of malignancies between wild type mice and *p53* double knock out mice, Norimura and co-workers have shown that the functionality of apoptosis in tumor resistance probably already starts prenatally (Norimura *et al.*, 1996). The resistance to radiation induced apoptosis in p53-/- mice is associated with an increase in embryonic survival compared to radiation sensitive wild type mice. Though, in later life the *p53-/-* population is characterized by a much higher incidence of malignancies, which is probably due to a limited deletion of damaged and potentially malignant cells shortly after *in utero* irradiation.

From this limited list of congenital malformations and diseases related to apoptosis, it is conceivable that this particular mode of cell death is a crucial component of animal existence. This notion is even further substantiated by the observation that fruit flies—*Drosophila melanogaster*—die early during embryogenesis when almost completely deprived of apoptosis by mutation or absence of genes in the apoptosis pathway, *i.e.*, *hid, grim* and *reaper* (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996). To further test the role of apoptosis in normal development and physiology on the one hand, and congenital malformations and disease on the other, it is essential to characterize this process biochemically and genetically (Table 1). Such knowledge may give rise to additional diagnostic tools regarding apoptosis related diseases, as well as means to modulate apoptosis as a part of treatment of disease.

Molecular biology of apoptosis

Programmed cell death

In addition to the term physiological cell death, apoptosis is also often referred to as programmed cell death. Initially this term was derived from the observation that this process occurs during development following highly reproducible patterns that were specified both in time and location, *i.e.* the program. At present, the term *programmed* is much more linked with the molecular regulation of this type of cell death, which is radically different from the passive demise of cells dying the necrotic way (Jacobson *et al.*, 1997).

Disease	Gene	Gene Map Locus	OMIM N°
Crouzon Craniofacial Dysostosis	CASP7 (candidate)	10q25-q25.2	601761
Holoprosencephaly	CASP2 (candidate)	7q35-q36	600639
Agammaglobulinemia	BTK	Xq21.3-q22	300300
Amyloidosis (Finish Type)	GSN	9q34	137350
Beckwith Wiedemann Syndrome	CDKN1C (candidate)	11p15.5	600856
Bladder Cancer	DAPK1 (candidate)	9q34.1	600831
Burkitt Lymphoma	MYC	8q24.12-q24.13	190080
Colorectal Cancer, T Cell Acute Lymphoblastic Leukemia	BAX	19q13.3-q13.4	600040
Congenital Dyserythropoietic Anemia Type 1	CDAN1	15q15.1-q15.3	224120
Epilepsy	CSTB	21q22.3	601145
Familial Amytrophic Lateral Sclerosis	SOD1	21q22.1	147450
Fanconi Anemia	MX1	21q22.3	147150
Follicular Lymphoma	BCL2	18q21.3	151430
Huntington Disease	HD	4p16.3	143100
Syndactyly Type I	Hox4 (candidate)	-	185900
Lympho Proliferative Syndrome (ALPS)	APT1/FAS	10q24.1	134637
Neurblastoma	TP73 (candidate)	1p36	601990
Paroxysomal Nocturnal Hemoglobinurea	PIGA	Xp22.1	311770
Familial Arrhythmogenic Right Ventricular Dysplasia 1	ARVD1	14q23-q24	107970
Spinocerebellar Ataxia 3	MJD	14q24.3-q31	109150
Spinomuscular Atrophy Type	SMN1	5q12.3-q13.3	600354
Stomach Cancer	BCL2A1 (candidate)	15q24.3	601056
Systemic Lupus Eruthematosis (SLE)	APT1LG1/FASL	1q23	134638
Various Tumors, e.g. lung, colon and skin carcinoma	TP53	17p13.1	191170
Waardenburgh-Shah Syndrome	SOX10	22q13	602229

Table 1: Genetic disorders possibly caused by disregulation of apoptosis [Online Mendelian Inheritance in Man (OMIM™). Center for Medical Genetics, John Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD),1997. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/]

Paradoxically, the species having contributed most to the understanding of the molecular biology of apoptosis is one that develops and survives under laboratory conditions in the absence of apoptosis without exhibiting gross abnormalities, the nematode *Caenorhabditis elegans* (Yuan *et al.*, 1993). By linking mutations in the genome of this nematode to an abnormal or absence of cell deletion, genes have been identified that are involved in various phases of apoptosis: activation, execution, phagocytic clearance and intraphagolysosomal degradation (Ellis *et al.*, 1986; Ellis and Horvitz, 1991b). These genes have been conserved by evolution, indicating the significance of apoptosis to the development and functioning of multicellular organisms. The signaling phase which precedes the activation of apoptosis is highly heterogeneous and is composed of a myriad of molecules and receptors which interact often in a species and cell type specific manner (for a recent review see: Vermes *et al.*, 1998).

The signaling phase of apoptosis

The signaling phase of apoptosis requires receptors at the cell surface to respond to the environment which may contain pro and anti apoptotic signals in soluble form or present on the surface of surrounding cells. In addition, it involves "sensor" mechanisms within the cell, to detect physical stimuli including cold, heat, and irradiation (Fesus, 1993; Akbar *et al.*, 1997). The cell type specificity of this signaling phase of apoptosis is illustrated by the observation that in *C. elegans* mutations that cause a gain of *ces1* function, or a loss of *ces2* function prevents apoptosis of specifically two cells in the pharynx (Ellis *et al.*, 1991a). Although it was not clear from this study how both genes effect such a cell specific action, it does show that the signaling phase consists of both apoptosis activating and apoptosis inhibiting cues. Such opposing cues do also exist in mammalian cell death. Some examples of rescuing signals include the growth hormones Fibroblast Growth Factor (FGF)-2 and FGF-4 (Macias *et al.*, 1996), Platelet Derived Growth Factor (Appleton *et al.*, 1993), as well the extracellular matrix protein fibronectin (Scott *et al.*, 1997). Some extensively studied apoptosis inducing factors include retinoic acid (Rizzo *et al.*, 1991), Fas ligand and its receptor (Los *et al.*, 1995), and the proto oncogene *c-myc* (Bisonette *et al.*, 1992; Fanidi *et al.*, 1992).

If after integrating the pro and anti apoptotic signals the balance shifts toward apoptosis, this message has to be transduced to the apoptosis activation-genes to initiate an apoptosis specific cascade of events. How this signal transduction is effected is only limitedly understood. But during this early phase of apoptosis, intracellular calcium normally rises (Trump *et al.*, 1995) and ceramide may be generated from sphingomyelin in the plasma membrane (Hannun *et al.*, 1995). Transduction of the message from the signaling level towards the level of apoptosis activation may entail macromolecular synthesis. This was most clearly shown in experiments in *Drosophila*, where transcriptional activation of the genes *reaper*, *hid* and *grim* is essential for cells to become apoptotic (White *et al.*, 1996). In addition, a recent investigation of a mammalian colorectal cancer cell line has indicated that induction of apoptosis by infecting these cells with a replication defective virus carrying the *p53* gene, was mediated by the activation of transcription of a number of redox related genes (Polyak *et al.*, 1997). However, exceptions to this rule apparently also exist. This was indicated by an elegant study of Fas induced apoptosis by Schultze-Osthoff and co-workers who showed that after enucleation, B cells still were able to execute the cell death program after being exposed to Fas ligand, as judged by the appearance of the morphological hallmarks of apoptosis like membrane blebbing and cytoplasmic condensation (Schultze-Osthoff *et al.*, 1994).

Apoptosis modulating agents which are cell type specific may possibly become part of therapeutic intervention in heart disease, diseases of the immune system, cancer, and neurodegenerative disorders. Such therapeutic drugs are most likely to be derived from the signaling level of the apoptotic cascade. Nevertheless, it should be taken into account that the same factors may act on different cell types, which is illustrated by the induction of cell death in the prospective neural crest (Graham *et al.*, 1994) and the interdigital mesoderm (Zou *et al.*, 1996) by Bone Morphogenetic Protein (BMP) during mouse embryonic development. In addition the same factor may transmit opposite signals, such as (1) endogenous NGF, which inhibits cell death of most neurons during development (Davies *et al.*, 1991; Albers *et al.*, 1994) while it activates cell death in the early central retina (Frade *et al.*, 1996), and (2) the inflammatory cytokine Tumor Necrosis Factor-α (TNF-α), which may inhibit apoptosis of B lymphocytes (B cells) in a dose

dependent manner up to 710 IU/ml (Mangan *et al.*, 1991) whereas it induces apoptosis and necrosis in, for example, the L929 line at a dose of 250 IU/ml (Piredda *et al.*, 1997).

The activation and execution phase of apoptosis

Numerous studies have shown that probably each cell contains all proteins that are required to activate and execute the apoptosis program, and that these molecules are constitutively expressed both in embryonic, fetal, neonatal and adult cells (Ishizaki *et al.*, 1995; Weil *et al.*, 1996). Thus, if the cell has reached the activation level of apoptosis, the program can no longer be interfered with by molecules like staurosporine which inhibits many protein kinases (Tamaoki *et al.*, 1990). However, the process is still active and requires ATP to continue (Richter *et al.*, 1996).

If after the integration of pro and anti apoptotic signals the cell has reached the apoptosis activation phase, the cell is not yet bound to die. Similar to the signaling level, also at this subsequent level both pro and anti apoptotic molecules compete for the final decision. Anti apoptotic molecules include the virus derived proteins p35 (Bump *et al.*, 1995) and CrmA (Tewari *et al.*, 1995), and members of the Bcl-2 family of proteins (Reed, 1994); the latter are homologous with *C. elegans* CED-9 (Hengartner, 1995). Interestingly, the Bcl-2 family includes both anti apoptotic members—Bcl-2α, Bcl-2β and Bcl-X—and pro apoptotic members—Bad, Bag and Bax—(Davies, 1995; Gonzáles-García *et al.*, 1995; Motoyama *et al.*, 1995; White *et al.*, 1998). The balance between hetero and homodimers of these pro and anti apoptotic proteins determines whether apoptosis is inhibited or activated (Reed, 1997).

Two mechanisms have been proposed by which the Bcl-2 family members may modulate apoptosis. First, Bcl-2 may inhibit the progress of apoptosis by blocking the release of molecules from the mitochondrion which include the apoptosis stimulating mitochondrial cytochrome C which is equal to Apoptotic Protease Activating Factor (Apaf)-2 (Liu *et al.*, 1996; Kluck *et al.*, 1997). Alternatively, and not mutually exclusive with the former option, Bcl-2 may bind Apaf1 which is homologous with *C. elegans* CED-4 (Vaux, 1997; Zou *et al.*, 1997). It is hypothesized that such binding would interfere with complex formation of Apaf1, Apaf2 and Apaf 3/caspase-9 (Li *et al.*, 1997) on the one hand, and pro-caspase-3 on the other hand, thus preventing the conversion of pro-caspase-3 into its active form (Jacobson *et al.*, 1997; Li *et al.*, 1997).

Caspase-3 stands on the border of the execution phase of apoptosis. This zymogen, which is homologous with the *C. elegans* CED 3 is also known under the synonym *the executioner*. Caspase-3 is a member of the family of caspases which in activated form cleaves substrates after specific aspartic acids (Barinaga, 1994; Los *et al.*, 1995). Since caspases are also able to cleave each other and thereby convert family members from zymogens into active enzymes, caspase activation can lead to parallel enzymatic pathways (Nagata, 1997). Some caspases exert their function at the level of activating apoptosis, which is upstream of caspase-3. Amongst these proteins is caspase-9, caspase-11 and caspase-1; the latter is the archetype caspase, formerly known as Interleukin (IL)-1β Converting Enzyme or ICE. Other caspases act downstream of caspase-3, at the apoptosis execution level. Combined, these pathways lead to a rapid breakdown of the cytoskeleton, lamin, organelles, and nuclear DNA. The latter is mediated by a protein named DFF, which is responsible for cutting the DNA into 300Kbp, 50Kbp and eventually into 180bp fragments (Wyllie, 1980; Oberhammer *et al.*, 1993). In addition, the protein poly(ADP-ribose)Polymerase (PARP) is cleaved; a protein which is suggested to function in DNA-repair and surveillance in its intact form (Nicholson *et al.*, 1995; Tewari *et al.*, 1995)

Adding to the complexity of the process of apoptosis, activation and execution pathways have been discovered that run parallel to the aforementioned molecular cascades. The alternative activation pathways include ubiquitin activated proteasomes—phylogenetically conserved multicatalytic complexes—which effect cellular changes characteristic for the execution phase of apoptosis, both directly and indirectly via activating members of the caspase family (Grimm *et al.*, 1996; Machiels *et al.*, 1996). Furthermore, in some cell types calpain is activated during the execution phase, which is also able to cleave DNA and PARP similar to caspases (Vanags *et al.*, 1996; Villa *et al.*, 1998).

Preparing the cell for efficient removal from tissues without disturbing the environment seems to be the major task for the execution machinery. To reach this end, digestion of intracellular components is initiated while the plasma membrane remains intact, whereas the spilling of the cellular contents to the environment is in many cell types further avoided by building a scaffold under the plasma membrane by the activation of tissue transglutaminases (Fesus *et al.*, 1989; Piredda *et al.*, 1997). In addition, relatively early during the execution phase the cell starts to expose characteristic molecules at its surface which signal its apoptotic state and lead to its recognition and removal by local phagocytes or specialized macrophages (Savill *et al.*, 1993).

The phagocytic clearance of apoptotic cells

Phagocytosis of apoptotic cells is a multistep process, involving recognition of the apoptotic cell and subsequent cell adhesion, engulfment and intraphagolysomal degradation. The relative importance of the phagocytic endpoint of apoptosis can probably best be read from studies of the molecular regulation of apoptosis in *C. elegans*. In this species, a limited number of genes are directing the activation (pro apoptotic *ced-4* ; anti apoptotic *ced-9*) and execution (*ced-3*) of apoptosis, and intraphagolysosomal degradation of apoptotic corpses (*nuc-1*), (Sulston, 1976; Albertson *et al.*, 1978; Ellis *et al.*, 1991a; Hengartner *et al.*, 1992). In contrast, the removal of apoptotic cells appears to be under a much more extensive genetic control and is regulated by two sets of genes, composed of *ced-2*, *ced-5* and *ced-10*, and *ced-1*, *ced-6*, *ced-7* and *ced-8*, respectively. Only specimens having a defective gene in both sets show loss of phagocytic capacity (Ellis *et al.*, 1991a), indicating that probably two independent pathways of phagocytic clearance of apoptotic cells exist in this species composed of a mere 1090 cells, of which a 131 die until it hatches.

Like many of the factors that mediate the previous phases of apoptosis, studies have shown that also the process of phagocytosis is partly under control of phylogenetically conserved genes. Recent data suggest that the extension of the phagocyte-plasma membrane during engulfment of apoptotic cells is mediated by the *C. elegans ced-5* gene which is homologous with *Drosophila* Myoblast City protein and human DOCK180 (Wu *et al.*, 1998). Furthermore, *C. elegans* Ced-7 is suggested to be homologous with the ATP binding cassette transporter ABC1, which is a 220kDa glycoprotein (Luciani *et al.*, 1996). Steric blockade of this putative anion transporter, greatly reduces the capacity of embryonic phagocytes to clear apoptotic cells. Thus ABC1 seems to be an important modulator of phagocytosis during development (Becq *et al.*, 1997). In addition, evolutionary distant species seem to share elements of the recognition phase of phagocytosis (see below).

The clean disposal of dying is crucial to the process of apoptosis. One means of effecting this is by removing dead cells before their plasma membrane integrity has become compromised, and the

cells begin to leak their potentially harmful contents to the environment (Savill *et al.*, 1989b). In addition to the rapid dynamics of the phagocytic process, the inertness of apoptotic cells with regard to their surroundings is further promoted by the release of anti-inflammatory substances and inhibition of the release of proinflammatory cytokines by the phagocyte after ingesting apoptotic material (Voll *et al.*, 1997; Fadok *et al.*, 1998; Savill, 1998). The proinflammatory cytokines that have been described in mammalian peripheral blood monocytes to be inhibited include IL-1β, IL-8, IL-12, granulocyte macrophage colony-stimulating factor (GMCSF), TNF-α, leukotriene C4 as well as thromboxane B2 (Voll *et al.*, 1997; Fadok *et al.*, 1998). Probably this inhibition is mediated via para and autocrine mechanisms involving the production of transforming growth factor (TGF)-β, prostaglandin (PG)E2, and platelet-activating factor (PAF) by the macrophage (Fadok *et al.*, 1998). Whereas the studies of Voll *et al.* (1997) and Fadok *et al.* (1998) show mainly overlapping data, they were conflicting with regard to the production of the anti-inflammatory cytokine IL-10. This may be explained by the different phagocytic triggers that were used in both studies, showing that IL-10-production may increase in the case of CD36 mediated phagocytosis (Voll *et al.*, 1997) or decrease after ingestion of immunoglobulin (Ig)G opsonized apoptotic cells (Fadok *et al.*, 1998).

The best characterized part of apoptotic cell removal is the phagocyte recognition of antigens which are specifically expressed at the surface of apoptotic cells. Most of the phagocyte receptors, and ligands expressed by the apoptotic cell have been identified in phagocytosis inhibition experiments using ligand like molecules and antibodies against the phagocyte receptor, or in by targeted disruption of the genes encoding a putative receptor or ligand. Receptor molecules exposed at the surface of phagocytes include the 61D3 antigen which is a single chain 75kDa protein identical to CD14 (Devitt *et al.*, 1998). Furthermore, scavenger receptors of the A type and B type appear to be involved in the clearance of apoptotic cells (Platt *et al.*, 1996). Most studied is the B type receptor CD36, which is part of a charge sensitive pathway of phagocytosis mediated by the glycoprotein thrombospondin (Savill *et al.*, 1989a; Ren and Savill, 1995a). In this pathway, thrombospondin bridges the dying cell and the phagocyte by binding a putative thrombospondin receptor and the CD36-vitronectin $\alpha_v\beta_3$ complex, respectively (Savill *et al.*, 1990; Ren and Savill, 1995a). Strongest support for a role of CD36 in the clearance of apoptotic cells comes from a study showing that the normally non-phagocytic COS cells become capable of ingesting apoptotic cells after transfection with CD36 (Ren *et al.*, 1995b). Data suggest that scavenger receptors probably also are important in the removal of apoptotic cells in *Drosophila* embryos (Abrams *et al.*, 1992), which include a member of the CD36 family called *croquemort*, *i.e.* catcher of death (Franc *et al.*, 1996). Other phagocytic pathways which have been elucidated by inhibition experiments in mammalian and amphibian systems involve lectin type interactions with carbohydrate structures and mannose/fucose sugars exposed at the surface of the dying cell (Duvall *et al.*, 1985; Dini *et al.*, 1992, 1995; Falasca *et al.*, 1996; Little *et al.*, 1996).

Also the distribution of the different classes of phospholipids across the plasma membrane bilayer has been shown to have a signaling function during the end of cellular existence. In viable cells the choline phospholipids, *i.e.* spningomyelin and phosphatidylcholine, are mainly residing in the outer leaflet of the plasma membrane. In contrast, virtually all the aminophospholipid molecules, *i.e.* phosphatidylserines (PS) and phosphatidylethanolamines, are hidden in the cytoplasm facing leaflet of the plasma membrane (Op den Kamp, 1979). This asymmetric distribution is maintained in an ATP (Seigneuret *et al.*, 1984; Zachowski *et al.*, 1989) and Mg^{2+}

(Bitbol *et al.*, 1987) dependent manner by so-called translocases. *In vitro* studies of mainly red blood cells and platelets, but also of apoptotic cells, have indicated that loss of this asymmetric distribution of phosphatidylserine may occur after activation of a phospholipid unspecific scramblase (Verhoven *et al.*, 1995; Basse *et al.*, 1996; Zhou *et al.*, 1997), in conjunction with inhibition of an aminophospholipid specific out-in translocase (Sune *et al.*, 1987; Zachowski *et al.*, 1987), and possibly by activation of an aminophospholipid specific in-out translocase (Gaffet *et al.*, 1995).

Two lines of evidence point towards an important role of cell surface exposure of PS in the triggering of phagocytosis: (1) *in vivo* clearance of erythrocytes by the reticuloendothelial system is positively related to the relative amounts of PS exposed at the plasma membrane of the red blood cell (Schroit *et al.*, 1985; McEvoy *et al.*, 1986; Schlegel *et al.*, 1987; Allen *et al.*, 1988), and (2) *in vitro* phagocytosis of leukocytes can be inhibited by adding liposomes to the cultures, only if these liposomes are containing PS (Fadok *et al.*, 1992a, 1992b; Flora *et al.*, 1994; Ashman *et al.*, 1995). There are data available suggesting that similar to other parts of the apoptosis machinery, also the functionality of PS exposure is conserved among species; insect phagocytes have been observed to recognize and ingest phospholipid vesicles composed of both phosphatidylcholine and PS, and not vesicles composed of phosphatidylcholine only (Ratner *et al.*, 1986). Some studies suggest that phagocyte recognition of PS exposing apoptotic cells may be mediated by a 94- to 97 kDa oxidized low density lipoprotein-receptor which is identical to mouse microsialin and homologous to human CD68 (Ottnad *et al.*, 1995; Ramprasad *et al.*, 1995; Sambrano *et al.*, 1995). In addition, phagocytosis of human apoptotic neutrophils *in vitro* is possibly mediated by binding of β2-glycoprotein 1 to the PS exposing cell (Balasubramanian *et al.*, 1997).

Taken together these studies of the process of removal of apoptotic cells indicate that multiple apoptosis specific surface antigens exist at the surface of apoptotic cells which are recognized by various phagocyte receptors and lead to engulfment and processing of the apoptotic cell. In addition to the listed routes for selective clearance of apoptotic cells, probably parallel apoptosis related receptor-ligand interactions will be identified in the near future, as is indicated by the identification of various specific epitopes at the surface of phagocytes and apoptotic cells with the use of monoclonal antibodies (Rotello *et al.*, 1994). In addition, adult T lymphocytes (T cells) show a number functionall changes of the plasma membrane including upregulation of the T-cell receptor-β/CD3, CD69 and CD25, whereas CD8 and CD4 are down-regulated after these cells are induced to undergo apoptosis by steroid treatment (Kishomoto *et al.*, 1995). As of yet, the function of these epitopes is still enigmatic.

Biochemical markers of apoptosis

The identification of molecular building blocks of apoptosis has led to the understanding that this organized mode of cell death is probably better characterized by its often phylogenetically conserved biochemical cascade, than by cell morphology only. In this respect, cells of the developing lens, healing cornea, and skin form good examples. The death of lens cells, keratocytes and keratinocytes is evidently an organized process which clearly differs from necrosis, but these dying cells do not show the morphological characteristics of apoptosis. Though, the identification of caspase-3 activity in cells of the lens and healing cornea, as well as

expression of tissue transglutaminases and the cutting of the genome into DNA fragments of 180 base pairs in keratinocytes during "terminal differentiation" has revealed that these cell death events are probably effected by mechanisms which are comparable to those found in "morphologically-correct" apoptosis (Polakowska *et al.*, 1994; Wilson *et al.*, 1996; Ishizaki *et al.*, 1998). It also sustains the notion that division of cell death into necrosis and apoptosis is still a valid working hypothesis. Furthermore, the appreciation that many of the biochemical components of apoptosis are shared between cells derived from all eukaryotes will probably give rise to new tools to detect apoptotic cells, which will help further deciphering the relation between apoptosis and normal development and physiology, as well as disregulation of apoptosis and congenital malformations and disease. This in turn may facilitate the identification of new factors in the signaling phase of apoptosis and the downstream effector molecules, leading to new insights and new markers. One of the downstream processes of the cell death machinery, that has already fulfilled a function in this respect for many years, is apoptosis associated internucleosomal DNA-fragmentation (Wyllie, 1980). Staining of gaps in the DNA of apoptotic cells via terminal deoxynucleotidyl transferrase-mediated incorporation of labeled deoxynucleotides has become a major tool to detect apoptosis both *in vitro* (Herrmann *et al.*, 1994) and *in vivo* (Gavrieli *et al.*, 1992; Wijsman *et al.*, 1993). Other more recent markers which have been derived from biochemical studies include antibodies raised against caspase-cleaved actin (Yang *et al.*, 1998) and activated caspase-3 (Kouroku *et al.*, 1998), as well as the probing for exposure of the phospholipid PS at the cell surface of apoptotic cells with the use of the PS binding protein annexin V (Koopman *et al.*, 1994; Martin *et al.*, 1995; Vermes *et al.*, 1995).

Annexin V

The protein family of annexins contains more than ten members, which have been isolated from a variety of eukariotes, including plants, invertebrates, fish, avian and mammals (Raynal *et al.*, 1994; Morgan *et al.*, 1997). Members of this phylogenetically old family share a tertiary structure consisting of four—or eight for annexin VI—highly conserved domains which are connected by linker peptides (Raynal and Pollard, 1994). Annexins are characterized by a high affinity for Ca^{2+} and the phospholipids present in cell membranes, *i.e.* phosphatidylcholine, sphingomyelin, phosphatidyl-ethanolamine and PS (Hoekstra *et al.*, 1993). The molecular and biochemical identity of annexins is determined by their N-terminal region with less than 5% amino acid-homology between the separate members (Morgan and Fernández, 1997).

The protein now known as annexin V was first discovered in 1979 (Bohn, 1979). In the subsequent decade this protein was also isolated by several other groups. The protein was given various names by different authors, which in chronological order include placental protein 4, 35K calelectrin, chromobindin 5, anchorin CII, vascular anticoagulant-α, calphobindin I, placental anticoagulant protein I, endonexin II, lipocortin V, and 35γ-calcimedin (for details see: van Heerde *et al.*, 1995). With regard to its phospholipid binding capacity, annexin V stands out from other annexins by its high affinity for PS (Tait *et al.*, 1988; Meers *et al.*, 1993). In addition to binding PS, annexin V has been shown to bind a number of other molecules, which include cytoskeletal proteins, collagen, and glycosaminoglycans (Webb *et al.*, 1987; von der Mark *et al.*, 1997; Ishitsuka *et al.*, 1998). Based on these binding properties, annexin V is attributed a variety of membrane associated functions which include the inhibition of blood coagulation, formation of calcium channels, stabilizing of phospholipid membranes, exocytosis and endocytosis (Goossens

et al., 1995; Larsson *et al.*, 1995; Kirch *et al.*, 1997; Rosales *et al.*, 1997), as well as inhibition of phospholipase A_2 (PLA_2) and protein kinase C (PKC) activity (Buckland *et al.*, 1998; Dubois *et al.*, 1998). Complicating the picture even any further, annexin V seems to be able to inhibit PLA_2 both dependent and independent of binding PS and Ca^{2+}, as has been shown by studies using model membranes (Speijer *et al.*, 1997) and annexin V derived peptides (Miele et al., 1988). Despite all its test tube functions, the roles of annexin V *in vivo* remain to be established.

Annexin V and the immune system

Although the *in vivo* function of annexin V is still enigmatic, one of the candidate functions of annexin V is modulation of the immune system. This is indicated by biochemical studies which have shown that annexin V-derived oligopeptides can exert anti-inflammatory effects *in vitro* and *in vivo*, including PGE2 release from plasma membranes and suppression IL-1β induced pyresis, respectively (Miele et al., 1988; Perretti et al., 1991; Douglas et al., 1992; Mugridge et al., 1993; Palmi et al., 1995). In addition, clinical studies have shown the presence of annexin V antibodies in patients with autoimmune diseases such as systemic lupus erythematosis (SLE) (Kaburaki et al., 1997) and rheumatoid arthritis (Dubois et al., 1995a; Rodriguez-Garcia et al., 1996), and in patients with recurrent pregnancy loss (Rand et al., 1994; Rand et al., 1997). In the latter, also frequently the normally present annexin V on the syncytiotrophoblast was reduced (Rand et al., 1994). Furthermore, in healthy persons annexin V levels have been found to be relatively high in immune privileged tissues such as the aforementioned allograft-placenta (Krikun et al., 1994), the eye (Kobyashi et al., 1990) and brain (Learmonth et al., 1992; Imai et al., 1995). If annexin V indeed has a role in modulating the immune system, one of its targets is possibly the B cell population. Cells of this lineage frequently play a role in diseases which seem to be linked with annexin V, including autoimmune diseases (Berek et al., 1997; Rudolphi et al., 1997; Eisenberg, 1998; Erikson et al., 1998; Ohtsuka et al., 1998), cell degeneration in normally immune privileged tissues (Sun, 1993; Owens et al., 1998) and allograft rejection (Hutchinson et al., 1995). More direct evidence of annexin V as an immunomodulatory molecule is, however, yet to be obtained.

Scope of this thesis and introduction to experimental work

The research presented in this thesis can be devided in two major sections. The first section pertains *the study of exposure of PS by apoptotic cells in vivo*. The tool used for this part of the thesis was the Ca^{2+} and phospholipid binding protein annexin V (Seaton *et al.*, 1990; Thiagarajan *et al.*, 1990; van Heerde *et al.*, 1995). Preceding the research presented in this thesis, in two studies it was shown that by its specific affinity for PS, recombinant human annexin V conjugated to a fluorochrome was a reliable marker for the detection of loss of the PS plasma membrane asymmetry by apoptotic neutrophils *in vitro*, *i.e.* it could discriminate between viable cells hiding PS inside the cell and apoptotic cells that exposed their PS molecules (Figure 2; Koopman *et al.*, 1994; Homburg *et al.*,

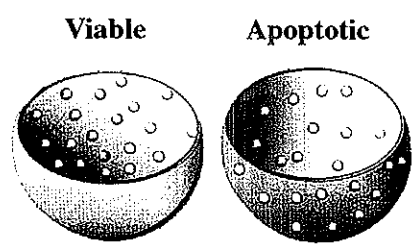

Figure 2: Schematic representation of the asymmetrical distribution of PS (small beads) in a viable cell. During apoptosis this plasma membrane asymmetry is lost, leading to cell surface exposure of PS which can be detected with annexin V.

1995). The first goal (**chapter 2**) was to test with annexin V, whether in embryos *in vivo* the distribution of PS across the two leaflets of the plasma membrane was as strictly regulated as in blood cells *in vitro*, and also to see if the regulation of PS plasma membrane asymmetry leading to cell surface exposure during apoptosis was a common phenomenon during mammalian development. Because PS is generally supposed to be present in all cell membranes, annexin V had to be introduced to the embryos in such a manner that it could only bind PS exposed at the outer plasma membrane leaflets. Therefore, annexin V conjugated to the marker-molecule biotin (annexin V-biotin) was injected into the circulation of viable mouse embryos from day 11-13 of development (E11-E13). After perfusing viable embryos for 30 minutes with the protein, specimens with positive heart activity were fixed, paraffin embedded and serially sectioned. Following, the spatial and temporal distribution of annexin V-biotin labeled cells was studied in these embryos at the light microscopical level, in particular with regard to previously documented regions of developmental cell death (Glücksmann, 1951). Furthermore, in "dense" regions of cell death at specific location in developing limb, we studied cellular binding of annexin V-biotin at the ultrastructural level.

Many components of the molecular machinery of apoptosis are shared between the various cell types that become apoptotic within species, and are conserved among species too. A phagocytosis inhibition study by Rattner and co-workers (Ratner *et al.*, 1986) using liposomes, indicated that PS exposure may be a membrane alteration utilized by insect hemocytes to clear apoptotic cells, similar to mammalian neutrophils. Based on this knowledge we tested the hypothesis that PS exposure by apoptotic cells is phylogenetically conserved (**chapter 3**). For this purpose Annexin V-biotin was injected intracardially into viable mouse and chick embryos, and into the hemolymph of *Drosophila melanogaster* pupae. The relation between annexin V-biotin labeling and apoptosis was studied at the spatiotemporal level as well as at the cell morphological level in paraffin and semithin sections.

Markers for apoptosis need to be specific and selective. In **chapter 4** the use of annexin V-biotin to label apoptotic cells was tested in comparison to other apoptosis markers in mouse embryos. These markers included the vital dye Nile blue sulfate, which labels superficial regions of apoptotic cell death in whole unfixed embryos (Saunders Jr *et al.*, 1962). It was also tested in this study how PS exposure and apoptosis associated DNA fragmentation were temporally related *in vivo*. This was done by a double labeling experiment for PS exposure and apoptosis associated DNA fragmentation on the degenerating interdigital tissue of E13 mice.

In **chapter 5**, a study is presented of neuronal cell death in early mouse embryos. Most literature regarding neuronal cell death during development derives from studies using avian embryos. But, at present the focus of research has started shifting towards genetical studies and mice have got into the picture. Therefore, apoptosis in the developing central nervous system and peripheral nervous system of E9-E14 mouse embryos was explored in a broad manner: the specificity and sensitivity by which annexin V bound apoptotic cells was evaluated, and histological- and quantitative- spatiotemporal cell death patterns were documented. In addition, an electron microscopical study was conducted, to investigate neuronal cell death at the subcellular level, and to compare phagocytosis of apoptotic neurons in peripheral ganglia with the phagocytic removal of dying mesodermal cells in the developing limb.

In contrast to the previous chapters, where human recombinant annexin V was used as a tool to study the distribution of PS exposing cells *in vivo*, the objective of the second section of this thesis was *to get insight in putative in vivo functions of annexin V*, with focus on modulation of the B cell immune response (**chapter 6**). The effect of human recombinant annexin V on isotype production by LPS or anti-CD40 polyclonally stimulated B cells was measured in conjunction with the effect of annexin V on cell proliferation and cell death. To get insight in the structure-function relationship of annexin V and its immunomodulatory effect, also mutant annexin V proteins with a decreased affinity for PS were tested.

Phosphatidylserine plasma membrane asymmetry *in vivo*: a pancellular phenomenon which alters during apoptosis

Stefan M. Van den Eijnde[1,5], Lenard Boshart[1], Chris P.M. Reutelingsperger[2], Chris I. De Zeeuw[3] and Christl Vermeij-Keers[3,4]

[1] Department of Cell Biology and Genetics, Institute of Clinical Genetics, Erasmus University Medical School, Rotterdam, The Netherlands
[2] Department of Biochemistry, Cardiovascular Research Institute, Limburg State University, The Netherlands
[3] Department of Anatomy, Erasmus University Medical School, Rotterdam, The Netherlands
[4] Department of Plastic and Reconstructive Surgery, Erasmus University Medical School, Rotterdam, The Netherlands
[5] corresponding author: S.M. Van den Eijnde, Department of Cell Biology and Genetics, Institute of Clinical Genetics, Erasmus University Medical School, PO BOX 1738, 3000DR Rotterdam, The Netherlands; tel: +31104087303; fax: +31104365780; email: vandeneijnde@ikg.fgg.eur.nl

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Abstract

The distribution of phospholipids across the two leaflets of the plasma membrane is important for many cellular processes including phagocytosis and hemostasis. In the present study we investigated the *in vivo* plasma membrane distribution of the aminophospholipid phosphatidylserine in mouse embryos with a novel technique employing Annexin V, a Ca^{2+} dependent phosphatidylserine binding protein, conjugated to fluorescein isothiocyanate and biotin. Annexin V directly applied to cryostat sections labeled the plasma membrane of all cells at the interface. In contrast, Annexin V injected intracardially into viable mouse embryos labeled almost exclusively apoptotic cells. These apoptotic cells were visible in all tissues and derived from all germ layers. Our experiments demonstrate that phosphatidylserine is asymmetrically distributed between the two leaflets of the plasma membrane in virtually all cell types *in vivo* and that this asymmetry is lost early during apoptosis.

Keywords: Annexin V, cell death, mouse embryo, phagocytosis, phospholipids

Abbreviations: AnxV, Annexin V; PM, plasma membrane; ABC, ATP-binding cassette; PS, phosphatidylserine; AnxV-FITC, Annexin V conjugated to fluorescein isothiocyanate; PC, Post coitum; LM, light microscopical; EM, electron microscopical; PBS, phosphate buffered saline; DAB, 3,3'-diaminobenzidine tetrahydrochloride

Introduction

In vitro studies mainly of blood cells have shown that the two major classes of the plasma membrane (PM) phospholipids, the choline- and aminophospholipids, are distributed asymmetrically between the two leaflets of the PM. Sphingomyelin and phosphatidylcholine comprise the majority of the outer leaflet while ethanolamine and serine phospholipids reside predominantly in the leaflet facing the cytosol (Devaux, 1991).

The PM transverse translocation of phospholipids is mediated by so-called flippases (Higgins, 1994; Diaz and Schroit, 1996). The first flippase has recently been identified. This phosphatidylcholine translocase is the *mdr2* p-glycoprotein gene product (Mdr2) (Smit *et al*, 1993), which is a member of the phylogenetically old, ATP-binding cassette (ABC) transporter superfamily (Higgins, 1992). In an ATP/Mg^{2+} dependent manner, this translocase flips phosphatidylcholine from the cytoplasm-facing leaflet to the opposite orientation (Ruetz and Gros, 1994). A similar machinery seems to regulate the phosphatidylserine (PS) distribution across the PM (Tang *et al*, 1996). A PS specific energy dependent out-in translocase activity (Martin and Pagano, 1987; Seigneuret and Devaux, 1984; Zachowski *et al*, 1989) was measured in membranes of anucleated (Seigneuret and Devaux, 1984) and nucleated (Devaux, 1991; Tang *et al*, 1996) cell types *in vitro*. For blood platelets it was shown that the PS translocase activity can be enhanced under conditions that give rise to the appearance of PS in the outer leaflet (Tilly *et al*, 1990). This regulatory mechanism provides a steady state with low levels of surface exposed PS (Diaz and Schroit, 1996).

In vitro studies have also indicated the existence of molecular machineries that counteract the above regulation causing an increase of PS in the outer leaflet of the PM (Diaz and Schroit, 1996). Receptor/ligand activated platelets (Bevers *et al*, 1983), and ageing erythrocytes (Connor *et al*, 1994) as well as apoptotic hematopoietic cell lines (Fadok *et al*, 1992a; Martin *et al*, 1995; Vermes *et al*, 1995) express PS at their cell surface while keeping PM integrity intact. The surface exposed PS catalyses reactions of the coagulation system (Bevers *et al*, 1982) and mediates recognition and uptake by phagocytes (Connor *et al*, 1994; Fadok *et al*, 1992a, b). Receptor/ligand activated platelets exhibit a scramblase activity with concomitant inhibition of the PS translocase and redistribute PS symmetrically over the two leaflets of the PM (Williamson *et al*, 1995). Both the translocase and scramblase activity are regulated by cytosolic Ca^{2+} levels (Williamson *et al*, 1995). Nucleated cells regulate PS asymmetry of the PM similar to platelets. During apoptosis of lymphocytes *in vitro* PS translocase is inhibited and the scramblase is activated (Verhoven *et al*, 1995). Recently the scramblase present in erythrocytes has been isolated and purified (Basse *et al*, 1996).

In the present study we have assessed the PS asymmetry of the PM *in vivo* in whole mouse embryos utilizing Annexin V. This protein is a member of the Annexin family of structural and functional related proteins (Van Heerde *et al*, 1995) and engages specifically with PS in a calcium-dependent manner through a putative binding

pocket for the serine headgroup (Swairjo *et al*, 1995). The presence of PS in cells was monitored by applying Annexin V conjugated to fluorescein isothiocyanate (AnxV-FITC) directly to cryostat sections of mouse embryos. The presence and extent of PS at the outer PM leaflet was assessed by intracardiac injection of biotinylated Annexin V (AnxV-biotin) into viable mouse embryos.

Results

All cells with exposed interior bind Annexin V

Applying AnxV-FITC to cryostat sections through limbs of day 13 mouse embryos showed that all cells contain Annexin V binding sites (Figure 1). The PM was especially intensely labeled. To a lesser degree, labeling was also observed at the nuclear membrane.

Distribution of intracardially administered Annexin V

AnxV-biotin was injected intracardially in vital mouse embryos such that the entire circulatory system was thoroughly perfused and widespread interstitial distribution was achieved. AnxV-biotin and heat inactivated AnxV-biotin, (i.e. AnxV-biotin with a destroyed phospholipid binding activity; Reutelingsperger *et al*, 1985), were administered to embryos of 11, 12 and 13 days post coitum (PC). While embryos injected with heat inactivated AnxV-biotin did not show any cell labeling, those that were injected with active AnxV-biotin showed cell labeling at specific locations in the whole organism (Figure 2).

Cells derived from all three germ layers were labeled at the many sites where cell death appears during morphogenesis (Glücksmann, 1951). Depending on embryonic age, AnxV-biotin binding was present with left-right symmetry and cranially in the more differentiated organs (Poelmann and Vermeij-Keers, 1976), e.g. eye (Figure 2a), in both the soma and axons of neurons of the central (Figure 2a, b) and peripheral (Figure 2c) nervous system, bronchi (Figure 2d), and caudally in primitive organs, e.g. somites and degenerating tailgut (Figure 2e) (Nievelstein *et al*, 1993). In addition, many labeled cells were observed in the degenerating interdigital tissue (Figure 2f).

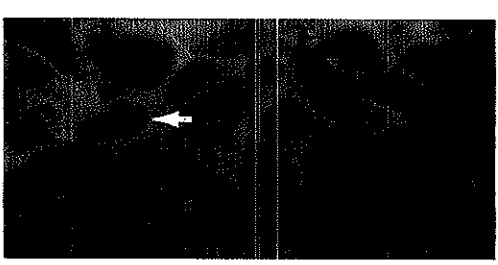

Figure 1 Cryostat section through day 13 mouse embryo limb, showing Annexin V binding sites in cells with exposed interior. AnxV-FITC binding was present in all cells, both at the nuclear membrane and PM (arrow).

Apoptotic cells bind Annexin V

The above results indicate that Annexin V binding cells are located in regions where cell death occurs during morphogenesis. To verify whether these cells were apoptotic, labeled cells in the interdigital mesenchymal tissue of day 13 mouse embryos were investigated for their ultrastructural characteristics.

AnxV-biotin positive cells showed the ultrastructural characteristics of apoptosis (Wyllie *et al*, 1980). Throughout the sections examples were found of AnxV-biotin labeled cells, undergoing phagocytosis including engulfment by neighboring cells, and more advanced stages of intraphagolysosomal degradation (Figure 3a and b). Other positive cells had more condensed chromatin (Figure 3c and d) and loss of electron density of the cytoplasm which contained an increased proportion of free ribosomes and dilated endoplasmatic reticulum while other organelles appeared unchanged (Figure 3c). The AnxV-biotin binding cells in later stages of apoptosis showed advanced chromatin condensation (Figure 3e), nuclear pyknosis (Figure 3f), and cell fragmentation.

Exceptions

In all the cases the Annexin V binding cells showed the morphology of apoptosis. However, two evidently non-apoptotic cell types also appeared to bind AnxV-biotin at their PM: (i) myoblasts differentiating into myotubes composed of a sarcolemma that closely ensheaths several aligned nuclei (Figure 4a) and myocardioblasts forming cardiac muscle (data not shown), and (ii) megakaryoblast and megakaryocytes in the liver, recognisable for their large size, polymorphic nucleus and the many thrombocyte-like attachments (Figure 4b).

Discussion

This paper demonstrates that regulation of PS topography and, consequently asymmetric membrane architecture, is a ubiquitous process during morphogenesis. All cell types confine PS to the cytoplasm-facing leaflets when viable. The process of apoptotic cell death results in PS exposure at the outer leaflet of the PM.

Annexin V is a protein that strongly binds to PS-containing membranes in the presence of Ca^{2+} (Van Heerde *et al*, 1995). Utilizing this capacity, *in vitro* studies indicated that Annexin V specifically marks apoptotic human neutrophils (Homburg *et al*, 1995), germinal B-lymphocytes (Koopman *et al*, 1994) and peripheral blood lymphocytes (Vermes *et al*, 1995). A study with hematopoietic cell lines and Annexin V demonstrated that these cells expose PS at the cell surface during apoptosis in culture, from an early stage onwards and regardless of the initiating stimulus (Martin *et al*, 1995).

We have applied Annexin V to study loss of PM asymmetry *in vivo*. Mouse embryos were used as a model, because they carry spontaneous, spatiotemporally consistent cell death patterns (Glücksmann, 1951). While all cells possess Annexin V binding sites, as we

demonstrated by cryostat sections, perfusion of viable embryos with AnxV-biotin revealed that only specific cell populations bear these binding sites at their cell surface. The interaction of AnxV-biotin with the embryonic cells relies on its phospholipid binding property, as was shown from our control experiment with heat inactivated AnxV-biotin.

Active AnxV-biotin identified cells at locations where cell death serves morphogenesis; in cells derived from all three germlayers, and both in primitive (Nievelstein *et al*, 1993; Vermeij-Keers and Poelmann, 1980) and in more differentiated tissues (Glücksmann, 1951; Poelmann and Vermeij-Keers, 1976). The whole cells were labeled, which was best visualized in dying neurons, with their labeling of both the soma and axons. At both the light microscopical (LM) and electron microscopical (EM) level, the AnxV-biotin labeled cells fitted into the sequence of morphological stages that apoptotic cells undergo (Poelmann and Vermeij-Keers, 1976; Wyllie, 1992; Wyllie *et al*, 1980), from the earliest stages, hardly distinguishable from viable neighboring cells, up to intraphagolysosomal degradation.

The consistent presence of PS on the outer layer of the PM of apoptotic cells and the common absence of PS exposure by viable cells suggests a tight regulation of PS PM asymmetry. This predicts an important physiological role for this process *in vivo*. One such a role may exist in the process of phagocytosis. It is this process that largely determines the elimination of apoptotic cells without any inflammatory reaction (Kerr *et al*, 1972; Martin *et al*, 1994; Savill *et al*, 1993). In effecting this, apoptotic cells must be recognized and ingested rapidly by phagocytes before membrane integrity is lost and the cells eventually succumb

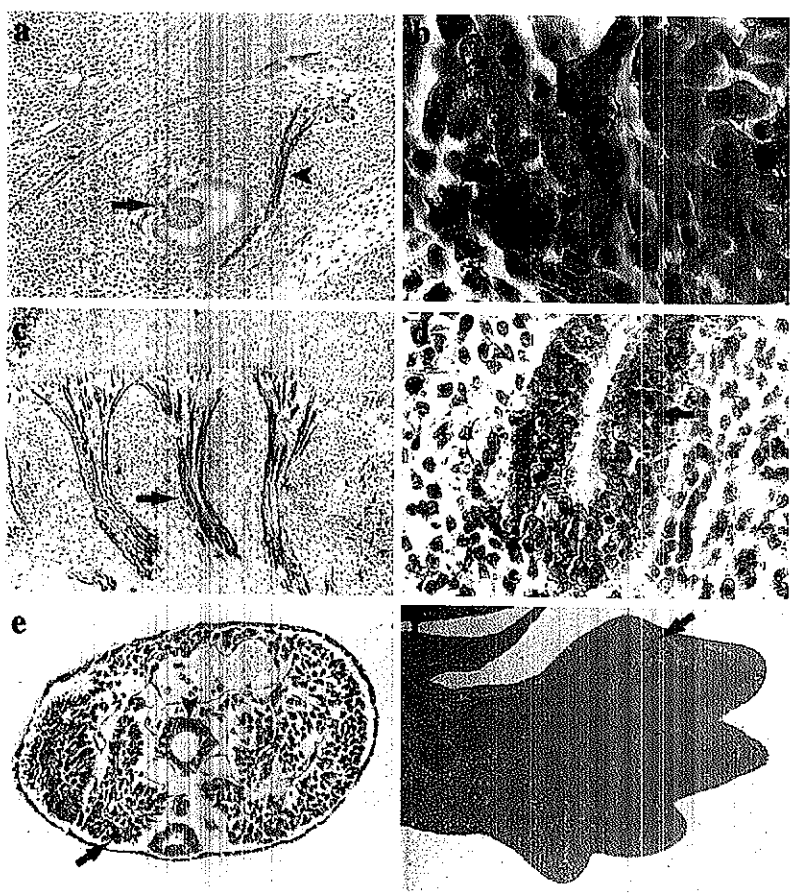

Figure 2 Examples of AnxV-biotin labeling in day 11 (a, b, d, e), day 12 (c) and day 13 (f) mouse embryos: eye (a: arrow), both the soma and axons of neurons of the central (a, b: arrowhead) and peripheral (c: arrow) nervous system, bronchi (d: arrow), somites (e: arrow) and degenerating tailgut (e: arrowhead). In between digits also the cells were marked (f: arrow).

to lysis (Majno and Joris, 1995; Wyllie, 1992). Exposure of PS after loss of PM asymmetry is one of three general structural changes in the PM of apoptotic neutrophils and lymphocytes that have been identified *in vitro*, together with formation of thrombospondin binding sites, and exposure of side chain sugars after loss of sialic acid (Savill *et al*, 1993). Like in the latter two, PS exposure may well permit recognition by phagocytes, which remove the dying cell by phagocytosis.

From our study it appears that the presence of PS at the outer PM marks the entire period of cell progression through apoptosis from the earliest stages, hardly distinguishable from viable cells, up to intraphagolysosomal degradation. The involvement of PS in phagocytosis of apoptotic cells may then explain why in *in vitro* phagocytic assays, next to phagocytes containing recognizable apoptotic cells, phagocytes have also been observed containing apparently viable cells (Savill *et al*, 1989). Moreover, it also explains the clearance of apoptotic cell fragments during morphogenesis. These cell fragments too still bear the surface structures necessary for recognition and removal by phagocytes.

In addition to apoptotic cells, two types of viable cells were observed to express Annexin V-binding sites at their cell surface. This may reflect a PS-dependent pathway for intercellular recognition shared by apoptotic cells and invoked by viable cells under specific conditions. The AnxV-biotin positive myoblast must promote homotypic recognition to fuse into myotubes and heart muscle syncytium. The AnxV-biotin positive megakaryocytes and megakaryoblasts may invoke the PS-dependent recognition pathway to keep the entities of already distinguishable platelet structure together until maturation is completed and platelets are dispersed. Within this concept of intercellular recognition an independently regulated but associated mechanism of phagocytosis should be considered. Whereas the apoptotic cells drive phagocytes to phagocytose them, myoblasts, megakaryocytes and megakaryoblasts should be inert in this respect or even discourage phagocytes to approach and phagocytose. Involvement of PS exposure in attachment of cells to each other without ending up in phagocytosis may indeed take place in certain circumstances, such as was observed in recent studies on the scavenger receptor present on mouse peritoneal macrophages. These macrophages bind erythrocytes exposing PS without subsequent engulfment (Ottnad *et al*, 1995; Sambrano and Steinberg, 1995).

In conclusion, our results indicate that confinement of PS to the inner leaflet of the PM is a ubiquitous process of

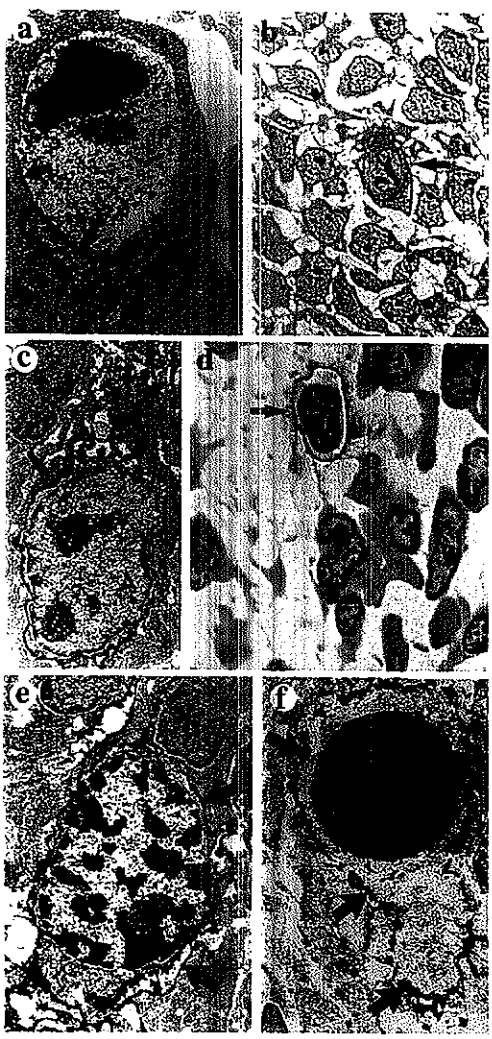

Figure 3 AnxV-biotin labeled cells (arrows) in day 13 mouse embryo interdigital mesenchyme in successive stages of apoptosis (EM: a, c, e, f; LM: b, d): ingested by phagocytes (a, b); early apoptotic, showing loss of electron density of the protoplasm, a dilated endoplasmatic reticulum (c: arrowhead; viable cell: asterix) and starting chromatin condensation (c, d); with advanced chromatin condensation (e) pyknotic, c.q. late apoptotic cell (f).

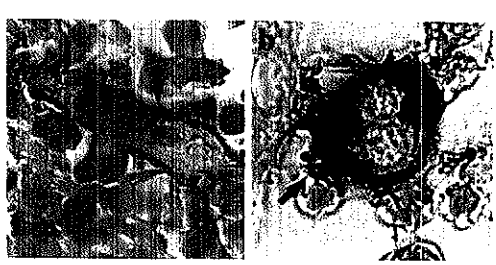

Figure 4 Annexin V labeled non-apoptotic cells (arrows) in paraffin embedded serially sectioned mouse embryos. (a) Section through a day 12 embryo showing membrane labeling of a myotube. (b) Section through a day 13 embryo showing a membrane labeled megakaryocyte.

viable cells *in vivo*. This PS PM asymmetry changes in surface exposure of PS early during apoptosis, irrespective from which germ layer the cells are derived. The general commitment of cells to this concept of regulation of PS PM topography makes it possible to visualize the many sites of apoptosis that are present during embryogenesis *in situ*, both at the topographical and at the ultrastructural level.

Materials and Methods

Experimental animals

Eighteen pregnant FVB-mice, from 11–13 days PC (plug=day 0), were killed by cervical dislocation after ether anesthesia. The uteri were dissected out and from the embryos collected, 116 were used for this study. From these embryos; nine were directly processed for cryostat sectioning and 107 were temporarily cultured for control experiments using heat inactivated AnxV-biotin (n=16) and for detection of loss of PS asymmetry of the PM by microinjection of AnxV-biotin (LM, n=54; EM, n=37).

Binding of FITC conjugated Annexin V to cryostat sectioned embryos

Day 13 mouse embryo limbs were sectioned on a cryostat (CM3000, Reichert Jung, Germany) at 10 μm. Directly thereafter, sections were washed with HEPES buffer, incubated for one minute with AnxV-FITC (APOPTEST^{TM}-FITC kit, a product from NeXins Research BV, The Netherlands), washed again with HEPES buffer and mounted with anti fading agent. The slides were stored at −4°C until examination. Sections were examined under a microscope using standard settings for FITC fluorescence-detection.

Heat inactivated Annexin V-biotin

AnxV-biotin was inactivated by heating it for 10 min at 56°C (Reutelingsperger *et al*, 1985). Microinjection and staining procedures were identical to those used for active Annexin V-biotin. Non-specific binding of Annexin V and quenching of endogenous peroxidase activity was tested for in this manner.

Microinjection of Annexin V-biotin

Embryos were perfused by microinjection using a Hamilton-Syringe pipetting system with glass needles (tip diameter ~20 μm). Per embryo, a volume of approximately 3 μl AnxV-biotin (660 μg/ml), purchased from NeXins Research BV, The Netherlands, (APOPTEST^{TM}-biotin kit), was injected through the ventricle of the heart under a surgical microscope while the embryo was kept in HEPES buffer (20 mM HEPES (pH 7.4), 132 mM NaCl, 2.5 mM $CaCl_2$, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, 0.5% BSA) at 37°C. When injected, a temporary blanching of the umbilical vein could be seen. Successfully injected embryos that showed heart activity after 30 min of incubation were fixed overnight in HEPES buffer containing 4% formalin at 4°C and further processed for LM.

Detection of Annexin V binding-biotin, LM

Following fixation embryos were dehydrated, embedded in paraffin and serially sectioned at 3 μm. Endogenous peroxidase activity was blocked by incubation in methanol/H_2O_2 (9 : 1 v/v) for 20 min. Sections were washed in phosphate buffered saline (PBS). Bound AnxV-biotin was visualized using the avidin-biotin complex method with horseradish peroxidase conjugated avidin (ABC Elite kit, Vector Laboratories, USA) at room temperature. After washing with PBS, staining was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (0.05%), and counterstained with Hematoxylin.

Detection of Annexin V-biotin binding, EM

Day 13 embryos were processed for EM. The embryos were dissected out of the uterus, microinjected with Annexin V as described above, and subsequently intracardially perfused with 0.5 ml 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. The limbs were removed, postfixed overnight in the same fixative, and cut on a Vibratome into 50 μm sections, which were processed to visualize the biotinylated Annexin V as described for LM. After the reaction with DAB, the sections were postfixed in 1.5% OsO_4 in a 8% glucose solution, rinsed in aquadest, stained *en bloc* in 3% uranyl acetate, dehydrated in dimethoxypropane and embedded in Durcupan (for details of EM procedures see: De Zeeuw *et al*, 1988, 1989). Semithin and ultrathin tissue sections were cut on an ultratome (Ultracut S, Reichert Jung, Germany); semithin and ultrathin sections were counterstained with Toluidine blue and with lead citrate, respectively. The ultrathin sections were examined in a Philips electron microscope (CM 10).

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Cell surface exposure of phosphatidylserine during apoptosis is phylogenetically conserved

S. M. van den Eijnde, L. Boshart, E. H. Baehrecke, C. I. De Zeeuw, C. P. M. Reutelingsperger and C. Vermeij-Keers

MGC Department of Clinical Genetics (S. M. van den Eijnde, L. Boshart), Department of Anatomy (C. I. De Zeeuw) and Departments of Anatomy, and Plastic and Reconstructive Surgery (C. Vermeij-Keers), Erasmus University Medical School, Rotterdam, The Netherlands; Center for Ag. Biotechnology, University of Maryland Biotech. Institute, USA (E. H. Baehrecke); Department of Biochemistry, Cardiovascular Research Institute Maastricht, University Maastricht, The Netherlands (C. P. M. Reutelingsperger)

Exposure of the aminophospholipid phosphatidylserine at the outer leaflet of the plasma membrane by apoptotic cells can trigger phagocytic removal of these dying cells. This functionality of phosphatidylserine exposure in the process of phagocytosis is indicated by *in vitro* studies of mammalian and insect phagocytes. We have studied the *in vivo* distribution of cell-surface exposed phosphatidylserine by injecting biotinylated Annexin V, a Ca^{2+}-dependent phosphatidylserine binding protein, into viable mouse and chick embryos and *Drosophila* pupae. The apparent binding of Annexin V to cells with a morphology which is characteristic of apoptosis and which was present in regions of developmental cell death indicates that phosphatidylserine exposure by apoptotic cells is a phylogenetically conserved mechanism.

Key words: Chick; development; *Drosphila*; mouse; phagocytosis; plasma membrane.

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Introduction

Apoptotic cells are ingested and digested by neighbouring cells or specialized macrophages, while retaining their intact plasma membrane (PM) and without eliciting any inflammatory response.[1] It is probably this silent disappearance which makes apoptosis a physiological kind of cell death. In *in vitro* studies of adult mammalian apoptotic neutrophils and lymphocytes, three kinds of structural changes of the PM have been identified that lead to phagocyte recognition: (1) formation of thrombospondin binding sites; (2) exposing side chain sugars after loss of sialic acid and (3) cell surface exposure of phosphatidylserine (PS).[2] Results from an *in vitro* study by Ratner *et al.*[3] have indicated that the involvement of PS exposure in cell removal may not be limited to mammalian species. Namely, like mouse peritoneal macrophages, insect phagocytes obtained from the moth *Heliothis virescens* specifically recognize and ingest phospholipid vesicles composed of both phosphatidylcholine and PS and not vesicles composed of phosphatidylcholine only. It was this study that prompted us to test whether the phenomenon of PS exposure on the PM of apoptotic cells *in vivo* is not restricted to mammals.

To test the hypothesis that the phenomenon of PS exposure on the PM of apoptotic cells is conserved among species, we have injected biotinylated Annexin V (AnxV-biotin) intracardially into mouse and chick embryos, and into the haemolymph of *Drosophila* pupae. The localization of this Ca^{2+}-dependent PS binding protein, which is indicative of cell surface exposure of PS was studied at the cellular level in semithin sections. The AnxV-biotin spatio-temporal binding patterns were evaluated in paraffin sectioned specimens.

Correspondence to S. M. van den Eijnde, MGC Dept. of Clinical Genetics, Erasmus University Medical School, PO Box 1738, 3000DR Rotterdam, The Netherlands. Tel: (+31) 10-4087303; Fax: (+31) 10-4362762; email: vandeneijnde@ikg.fgg.eur.nl

Materials and methods

AnxV-biotin binding in mouse embryos

Experimental animals. Pregnant FVB-mice, from 10–14 days post coitum (plug = day 0), were sacrificed by cervical dislocation after ether anaesthesia. The uteri were dissected out, and of the embryos collected, 60 were used for detection of cell surface exposed PS by microinjection of AnxV-biotin according to procedures recently described by van den Eijnde *et al.*[1] Another 16 embryos were used for control experiments using AnxV-biotin which was inactivated by heating it for 10 min at 56°C.[1] Non-specific binding of Annexin V and quenching of endogenous peroxidase activity was tested for in this manner.[1]

Microinjection of AnxV-biotin. Embryos were perfused by microinjection using a Hamilton-Syringe pipetting system with glass needles (tip diameter ~ 20 µm). Per embryo, a volume of approximately 3 µl AnxV-biotin (APOPTEST™-biotin B-500; NeXins Research BV, Hoeven, The Netherlands), was injected through the ventricle of the heart under a preparation microscope while the embryo was kept in HEPES buffer (20 mM Hepes (pH 7.4), 132 mM NaCl, 2.5 mM $CaCl_2$, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, 0.5% BSA) at 37°C. When injected, a temporary blanching of the umbilical vein could be seen. Successfully injected embryos that showed heart activity after 30 min of incubation were fixed and further processed for light microscopy (LM).

Detection of AnxV-biotin binding. The morphology of AnxV-biotin binding cells was studied in semithin sections through day 12 embryos. To obtain these sections, embryos were microinjected with AnxV-biotin as described above. After removing the placenta, the embryos were subsequently intracardially perfused with 0.5 ml 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. The limbs were removed, postfixed overnight in the same fixative, and cut on a Vibratome into 50 µm sections. Endogenous peroxidase activity was blocked by incubating the sections in methanol/H_2O_2 (9:1 v/v) for 20 min. Sections were washed in phosphate buffered saline (PBS). Bound AnxV-biotin was visualized using the avidin-biotin complex method with horseradish peroxidase conjugated avidin (ABC Elite kit, Vector Laboratories, USA) at room temperature. After washing with PBS, staining was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (0.05%). After the reaction with DAB, the sections were postfixed in 1.5% OsO_4 in a 8% glucose solution, rinsed in aquadest, stained en bloc in 3% uranyl acetate, dehydrated in dimethoxypropane and embedded in Durcupan. Semithin tissue sections were cut on an ultratome (Ultracut S, Reichert Jung, Germany) and counterstained with Toluidine blue.

Day 10–14 mouse embryos were used for the study of AnxV-biotin binding patterns. These embryos were fixed overnight in HEPES buffer containing 4% formalin at 4°C, dehydrated and embedded in paraffin and serially sectioned at 3 µm. The sections were processed to visualize the biotinylated Annexin V as described for the semithin sections and were counterstained with Hematoxylin.

AnxV-biotin binding in chick embryos

Eggs from white leghorn chickens, obtained from Drost, Loosdrecht, The Netherlands, were incubated at 37°C, 80% relative humidity. The eggs were removed from the incubator when the embryos were expected to have reached the Hamburger Hamilton (HH) stage 18.[6] After windowing the eggs, the embryos were staged. Embryos at HH stage 17–19 were intracardially injected with AnxV-biotin (n=10) or heat inactivated AnxV-biotin (n=4) and incubated for 30 min, at 37°C. After incubating, the embryos were removed from the egg, and processed for AnxV-biotin visualization in paraffin sections as described above.

AnxV-biotin binding in *Drosophila* pupae

Drosophila melanogaster larvae were collected when they had reached the prepupal stage.[7] After 30 h of incubation at 22°C, the pupae at stage P5–P7 were removed from the cuticle under a preparation microscope. At room temperature, pupae were microinjected with 1 µl AnxV-biotin (1 mg/ml) (n=20) or heat inactivated AnxV-biotin (n=6) into the haemolymph. After 2 h of incubation at room

temperature, the pupae were rinsed with HEPES buffer and subsequently processed for AnxV-biotin visualization. The procedures were similar to those used for the mouse embryos except for exchanging a 100% ethanol for pentane/100% ethanol (1:1 v/v) as the last step of dehydrating the specimens.

Results

While the mouse and chick embryos and *Drosophila* pupae that were injected with heat inactivated AnxV-biotin (*i.e.*, AnxV-biotin with a destroyed phospholipid binding activity[4,5]) did not show any cell labelling, those that were injected with active AnxV-biotin showed cell labelling at specific locations.

AnxV-biotin binding in mouse embryos

Semithin sections. In semithin sections through day 12 mouse embryos, AnxV-biotin was observed to bind specifically to the PM of cells that were showing the features of apoptosis. Compared to the surrounding viable cells, the labelled apoptotic cells were more rounded-off and also had a more condensed chromatin structure (Figure 1A) reaching up to clear pyknosis (Figure 1B). In addition, such early and late apoptotic cells that were AnxV-biotin positive had been ingested by phagocytes. Figure 1C shows an example of a labelled phagocytosed cell that appears to be in the early phase of apoptosis, exhibiting only the first signs of chromatin condensation.

Paraffin sections. In the paraffin embedded and serially sectioned mouse embryos, AnxV-biotin labelling was observed at specific locations throughout the embryos. Examples of such spatial and temporal specific cell death patterns[6] visualized with AnxV-biotin staining are given in Figure 2. In day 10 embryos cell death is shown in the developing lens vesicle (Figure 2A) and in the fusing maxillary and nasal prominences (Figures 2B1–B2). In Figure 2C examples are presented from day 11 mouse embryos. AnxV-biotin labelling is shown in Rathke's pouch, *i.e.*, the presumptive pharyngeal pituitary, as well as in the ectoderm of the stomodeum directly underneath Rathke's pouch, and in the adjacent neuro-epithelium of the infundibulum. Endoderm-derived apoptotic cells were present in the thymus anlagen (Figures 2D1 D2). In day 13 metanephros (Figure 2E) apoptosis is located in areas of epithelial mesenchymal interaction and in the developing nephrones. In Figures 2F1–F2 labelled cells are shown in the day 14 thymus.

AnxV-biotin binding in chick embryos

In accordance with our findings in mouse embryos, the AnxV-biotin binding cells in the chick were located in regions where apoptotic cell death serves developmental processes. Examples are shown staining in the fusion area of the maxillary and mandibular prominences in an HH17 embryo (Figures 3B1–B2) and in the aortic valve leaflet of an HH19 embryo (Figures B1–B2).

Figure 1. Semithin sections through day 12 mouse embryo showing early (A; arrow) and late (B; arrow) apoptotic cells that are AnxV-biotin positive at the PM, amidst unlabelled viable cells. Also, labelled cells were phagocytosed (C; arrow).

AnxV-biotin binding in *Drosophila* pupae

Examples of AnxV-biotin stained cells in areas where apoptotic cells are present during *Drosophila* pupariation are depicted in Figure 4. Labelled pyknotic cells (compare with Figure 1B) are shown in the proboscis of a P5 pupa (Figures 4A1–A2). Similar to our findings in mouse embryos (see Figure 1C) labelled apoptotic cells were found ingested by phagocytes/haemocytes in *Drosophila* (P6: Figure 4B). Figures 4C1–C2 show an intense labelling of cells in a stage P7 *Drosophila* eye. The timing of this is consistent with previously described developmentally regulated cell death in the eye.[9]

Discussion

After receiving the signal to die, or after loss of the stimulus to survive, an energy consuming death machinery is initiated in the cell that leads to apop-

Figure 2. Paraffin sections through day 10 (**A, B1–B2**), day 11 (**C, D1–D2**), day 13 (**E**) and day 14 (**F1–F2**) mouse embryos. Examples are shown of AnxV-biotin binding cells in the lens vesicle (**A**), both in the epithelium of the developing lens (arrow), and in its lumen (arrowhead), the overlaying ectoderm is marked with an asterix. At another location in the same day 10 embryo (**B1**; overview), AnxV-biotin has stained cells (**B2**; arrow) at the fusion site of the maxillary (**B2**; max) and nasal prominences (**B2**; np). In a one-day-older embryo, AnxV-biotin binding cells are shown in the developing pituitary (**C**; t = telencephalon, d = diencephalon); cells were stained in the epithelium of the pharyngeal pituitary (**C**; arrow), in the underlying ectoderm of the stomodeum (**C**; asterix) and in the future posterior pituitary (**C**; arrowhead). Directly above the heart in another day 11 embryo (**D1**; p = pericardial cavity) cells were labelled in the developing thymus (**D2**; arrow). Note the labelled cells in the myocardial wall of the atrium (**D1**; arrowhead), indicating the site of injection of AnxV-biotin. In day 13 metanephros, cells were stained in the developing glomeruli (**E**; arrow) and at the epithelial-mesenchymal interface (**E**; arrowhead). Figure **2F** shows cells in a day 14 thymus (**F1**; overview, p = pericardial cavity), at a higher magnification pyknotic labelled cells could be recognized (**F2**; arrow).

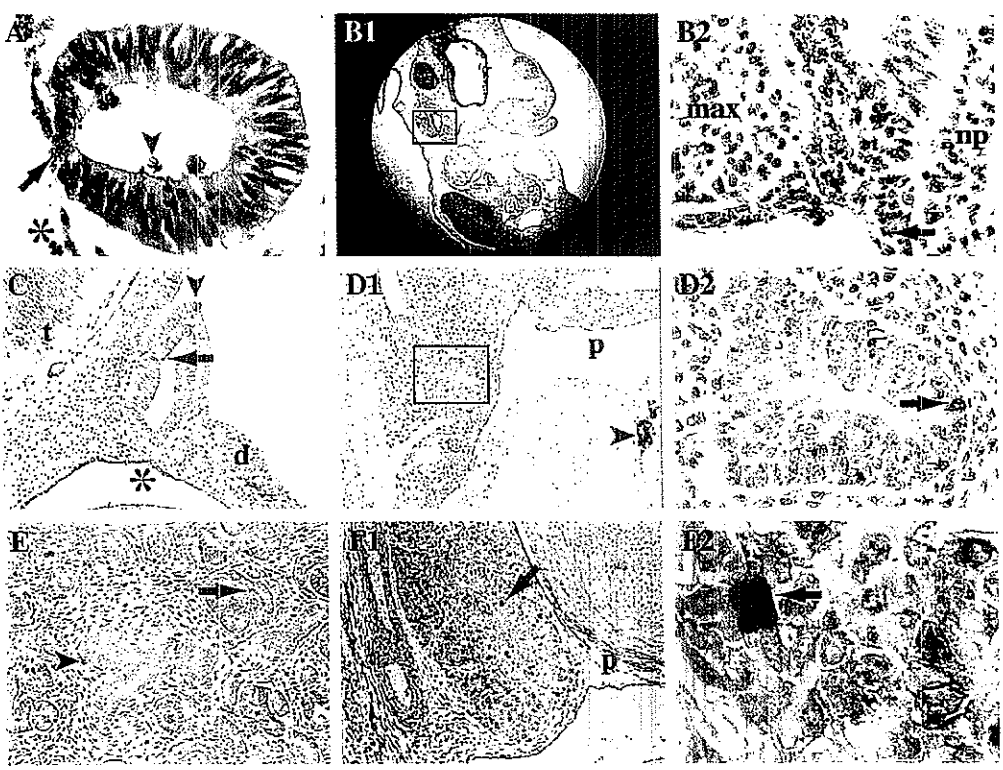
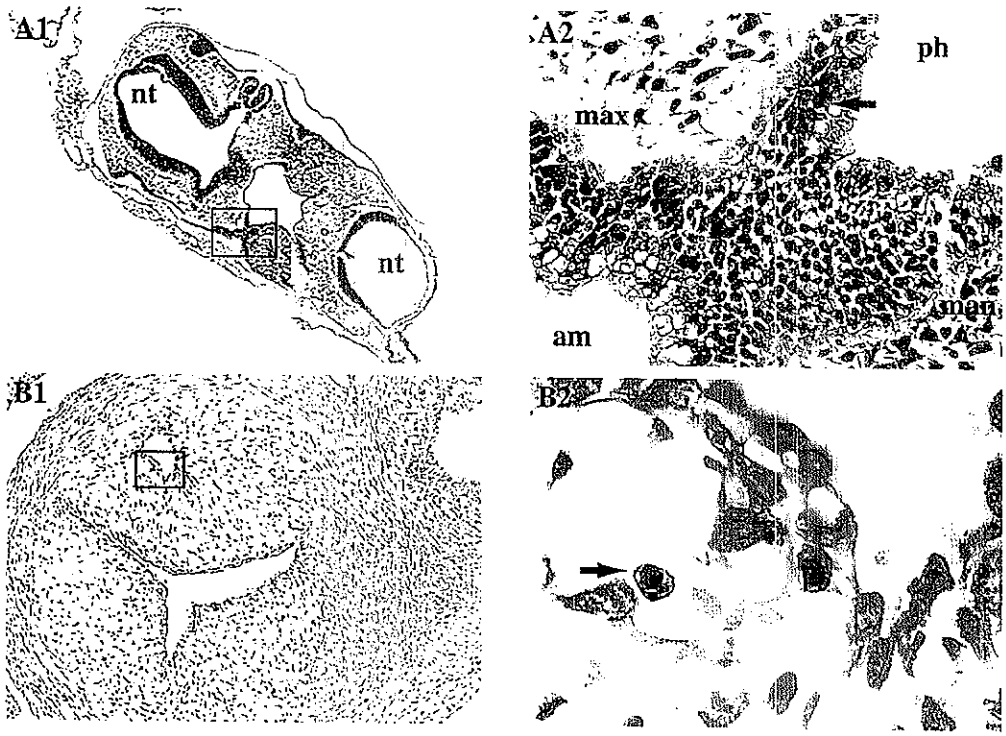


Figure 3. Examples from chick, showing labelled cells in the head region of an HH16 embryo (A1; overview, nt = neural tube) where apoptosis functions in the fusion of the maxillary (A2; max) and mandibular prominences (A2; man, ph = pharynx, am = amniotic cavity). Figure 3B shows cell death in an aorta valve leaflet of a stage HH18 embryo (B1; overview). At a higher magnification the labelled cells were seen to be pyknotic (B2; arrow).

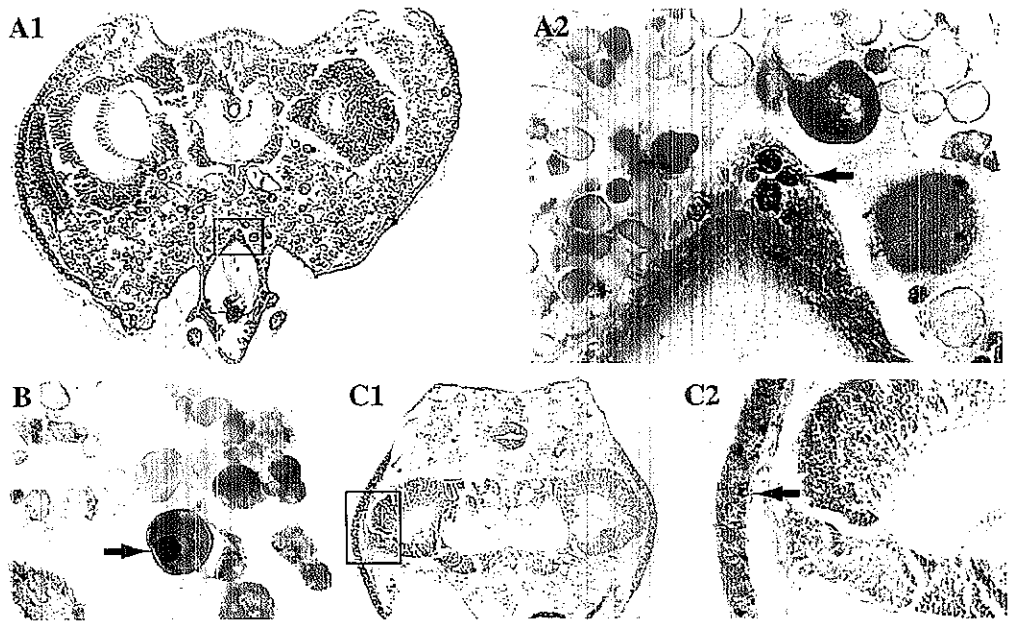


tosis[10,11] This machinery entails biochemical and morphological changes in the nucleus[12–14] and the cytosol.[1,15] The PM also plays an active role in the process of apoptosis by expressing specific epitopes[16] that may trigger the phagocytic removal of the cell by phagocytes.[2] One such an epitope is the PS molecule.[17–20]

Phospholipids are not equally distributed across the two leaflets of the PM. In viable cells, aminophospholipids like PS are mainly located in the PM leaflet that faces the cytosol. This PS asymmetry of the PM is achieved by the action of so-called flippases,[21,22] which translocate PS from the outer PM leaflet to the inner layer in an ATP and Mg^{2+} dependent manner.[23–26] Under specific conditions, the PS out-in translocase(s) are inhibited and a PS scramblase is activated, leading to a symmetrical distribution of PS across the two leaflets of the PM, effecting cell surface exposure of PS.

In *in vitro* assays, PS exposure at the outer PM leaflet has been described to occur in receptor/ligand activated platelets,[27] ageing erythrocytes,[28,29] and during apoptosis in a variety of cell types, *i.e.*, smooth muscle cells,[30] spermatogenic cells[19] and blood cells.[30–33] Recently we have established that PS–PM asymmetry is also tightly regulated in the developing day 11–13 mouse embryo *in vivo*. Through intracardial injections using AnxV-biotin as a probe[4,34] we have shown that binding of this Ca^{2+} dependent PS binding protein was mostly restricted to apoptotic cells. Apoptotic cells throughout the embryo appeared to carry PS in their outer PM leaflet, irrespective of the cell's lineage. In line with the *in vitro* studies,[35] the cells binding AnxV-biotin were in the process of apoptosis, from early stages when the apoptotic cells were only

Figure 4. Examples from insect showing labelled apoptotic cells in the proboscis of a stage P5 *Drosophila* melanogaster pupa (A1; overview), at a higher magnification these AnxV-biotin cells were observed to be pyknotic (A2; arrow). A haemocyte in a P6 pupa that has ingested a labelled and presumably apoptotic cell (B; arrow) is depicted in figure B. In a transverse section through a P7 pupa (C1; overview) cells are intensely labelled in the eye. This is shown at a higher magnification in Figure C2.



distinguishable from viable cells at the electron microscopical level, until after fragmentation into apoptotic bodies.[4] Furthermore, this PM alteration appeared to precede apoptosis associated DNA-fragmentation as detected with the TUNEL procedure.[34]

In the present study we have examined PS exposure in day 10–14 mouse embryos, but of greater importance, we have also determined whether apoptotic cells from avian and from non-vertebrate species expose PS, like their mammalian counterparts. The presence and extent of PS at the outer PM leaflet was assessed by intracardial injections of biotinylated AnxV-biotin in mouse and chick embryos, and *via* injection into the haemolymph in *Drosophila* pupae. This study was conducted in developing animals because of their reproducible, strictly spatio-temporally regulated apoptosis patterns, which facilitate the evaluation of the Annexin V binding patterns. The interaction of AnxV-biotin with the embryonic/pupal cells relies on its phospholipid binding property, as was shown from absence of labelling in the species that had been injected with heat-inactivated AnxV-biotin, *i.e.*, Annexin V with a destroyed phospholipid binding capacity.[4,5]

In mouse embryos, in accordance with previous observations,[4,34] AnxV-biotin appeared to bind to apoptotic cells in early and late apoptotic stages, as well as to cells that were in the process of becoming phagocytosed. In addition to the study of cell morphology in semithin sections, we also studied the Annexin V binding patterns in paraffin embedded serially sectioned mouse embryos. Specific patterns of AnxV-biotin labelling were observed in tissue areas where cell death serves developmental processes, throughout the embryos. This labelling appeared to be independent of the cell's lineage, *i.e.*, ectoderm, endoderm and mesoderm. AnxV-biotin positive cells were also observed among a subpopulation of circulating cells (data not shown). We consider these large embryonic red blood cells

with a pyknotic and often fragmented nucleus as early erythroblasts derived from the yolk sac. This cell type is destined to be removed in embryos that are in the transitional phase from a nucleated to an anucleated red blood cell population.[16] The embryos under study were in this transitional period. Both the adherence of these cells to endothelial cells as well as the presence of many cell fragments in endocardial cells and endothelial cells throughout the embryos suggest that these embryonic red blood cells expose PS similar to adult ageing erythrocytes[17] that are cleared from the circulation. However, the exclusive clearance of adult aged red blood cells by specialized endothelial cells of the reticuloendothelial system[38,39] probably does not hold for the embryonic counterparts.

In chick embryos and *Drosophila* pupae, we observed specific binding of AnxV-biotin to cells in regions where apoptosis plays a developmental role, similar to our findings in mouse embryos. The morphology of AnxV-biotin binding cells could be evaluated in the paraffin sections to some extent. Often AnxV-biotin appeared to have bound to cells that were pyknotic or fragmented. We consider this as a further evidence of the binding of AnxV-biotin to apoptotic cells in these species, in addition to the observed AnxV-biotin binding patterns during development.

On the basis of Annexin V's specific *in vivo* binding to apoptotic cells *in vivo* during mouse, chick and *Drosophila* development, we conclude that the regulation of PS asymmetry is shared by mammals, avians and insects and appears to be a phylogenetically primitive mechanism of cellular existence. The general commitment of cells to expose their PS molecules to the outer layer of the PM when they become apoptotic leads to the assumption that it is an important factor in phagocytic removal in the developing mammal, avian and insect. However, conclusive evidence for such a role, as has been given for adult mammalian cells[18–20] has yet to be established.

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In Situ Detection of Apoptosis During Embryogenesis With Annexin V: From Whole Mount to Ultrastructure

Stefan M. van den Eijnde,[1]* Antonius J.M. Luijsterburg,[3] Lenard Boshart,[1] Chris I. De Zeeuw,[2] Jan Hein van Dierendonck,[4] Chris P.M. Reutelingsperger,[5] and Christl Vermeij-Keers[2,3]

[1]MGC Department of Clinical Genetics, Erasmus University Medical School, Rotterdam, The Netherlands

[2]Department of Anatomy, Erasmus University Medical School, Rotterdam, The Netherlands

[3]Department of Plastic and Reconstructive Surgery, Erasmus University Medical School, Rotterdam, The Netherlands

[4]Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

[5]Department of Biochemistry, Cardiovascular Research Institute, University Maastricht, The Netherlands

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Apoptosis is of paramount importance during embryonic development. This insight stems from early studies which correlated cell death to normal developmental processes and now has been confirmed by linking aberrant cell death patterns to aberrant development. Linking apoptosis to the phenotype of a developing organism requires spatial information on the localization of the dying cells, making in situ detection essential. This prerequisite limits the tools available for such studies (1) to vital dyes, which can be detected at the whole mount level only; (2) to detection based upon apoptotic morphology by routine light microscopy and electron microscopy; and (3) to staining for apoptosis associated DNA fragmentation via, e.g., the TUNEL procedure, which marks cells in a relative late phase of apoptosis. New apoptosis markers need to be specific and should preferebly detect cells early during this process. In the present study we show that the recently discovered in vitro marker of apoptosis, Annexin V meets these requirements for in vivo detection. Through intracardiac injections of biotin labeled Annexin V, a Ca^{2+} dependent phosphatidylserine binding protein, we were able to visualize apoptotic cells derived from each germ layer in the developing mouse embryo from the whole mount level up to the ultrastructural level. Double-labeling on paraffin sections for both this method and TUNEL revealed that cells become Annexin V-biotin labeled early during the process of apoptosis. Cytometry 29:313–320, 1997.

Key terms: cell death; morphogenesis; mouse embryo; phagocytosis; phosphatidylserine; phospholipids; TUNEL

Cell death as a part of life was first recognized in studies of embryonic development from over a 150 years ago (5). In a thorough review on this subject, Glücksmann listed many of the spatiotemporally changing and species-specific cell death patterns that are present during development (13). In addition, he described the changes in cell morphology accompanying this type of cell death, which consists of nuclear and cytoplasmatic condensation, and fragmentation into membrane bound vesicles, sometimes containing dense nuclear material. Similar morphological changes, which were observed in a model of atrophy of adult liver, formed the basis for the definition of apoptosis (17). This specific morphology not only enabled microscopical detection of apoptotic cells but also allowed their distinction from necrotic cells, which are characterized by loss of staining of the nucleus and early degenerative changes of the mitochondria (50).

A functional difference between necrotic and apoptotic cells is that the latter are removed by phagocytes and degraded in phagolysosomes, without eliciting an inflammatory response (6,35,50). To be removed, the apoptotic cell has to signal its death to the environment. Phagocytes pick up this signal and respond by engulfing the apoptotic cell before it loses the integrity of its plasma membrane (35,50). Recent in vitro studies have indicated that the aminophospholipid phosphatidylserine (PS), which normally resides in the cytoplasm facing leaflet of the plasma membrane (9), serves as such a signal when it becomes

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*Correspondence to: Stefan M. van den Eijnde, MGC Department of Clinical Genetics, Erasmus University Medical School, P.O. Box 1738, 3000DR Rotterdam, The Netherlands.

E-mail: vandeneijnde@ikg.fgg.eur.nl

expressed on the outer surface of the apoptotic cell (2,10,11,37).

The PS exposed by the plasma membrane of apoptotic cells can be detected by using Annexin V. This 35 kDa protein is a member of the Annexin family (31,33) and has a high affinity for PS containing membranes after binding Ca^{2+}-ions (1,36,39,43). Probing for apoptotic cells with Annexin V in vitro has shown that the cell surface exposure of PS is an early event preceding nuclear changes (4,23), occurring while the integrity of the plasma membrane is still uncompromised (16,20,47). Furthermore, PS exposure appears to be ubiquitous among hematopoietic lineages, occurring irrespective of the apoptosis initiating stimulus (23). Recently it has been demonstrated that apoptotic adherent cell types in culture (3,42) and tissue embedded cells (40) also expose PS at their cell surface.

Reliable means to detect apoptosis are necessary to study the (patho)physiology of this process during development. Appropriate in situ markers should facilitate both the identification of the spatial patterns and the cytological/histological characteristics of the apoptotic cells in a selective and sensitive manner. In the present study we have tested the feasibility of Annexin V as an apoptosis marker in embryonic tissues both at the whole mount level and in paraffin and ultrathin sections. Using biotinylated Annexin V (AnxV-biotin) apoptosis was measured in the intact living mouse embryo. Day 11-13 mouse embryos were the model of choice, since these carry spontaneous, highly consistent spatiotemporal apoptosis patterns that have been documented extensively, especially for the limbs (18,19,24,26,41,51).

MATERIALS AND METHODS

Embryos

Pregnant FVB-mice, from 11-13 days PC (plug = day 0), were sacrificed by cervical dislocation after ether anesthesia. The uterus was taken out and embryos were collected and temporary cultured for detection of apoptosis using Nile blue sulfate (n = 35) or for detection of cell surface exposed PS using AnxV-biotin [whole mount, n = 32; light microscopy (LM), n = 95; electron microscopy (EM), n = 37]. Eight of the AnxV-biotin injected paraffin-sectioned embryos were used for double labeling experiments, consisting of in situ visualization of both AnxV-biotin binding and apoptosis associated DNA fragmentation via the TUNEL assay for 3'-hydroxyl DNA ends (12). Embryos injected with heat inactivated AnxV-biotin (n = 16) served as controls.

Nile Blue Sulfate

After removal of the extra embryonic membranes, embryos were stained in toto for 30 minutes at 37°C in a Nile blue sulfate solution (1:20000 w/v) (34). After incubation, the specimens were rinsed twice with phosphate buffered saline (PBS), put on ice, and immediately photographed under a microscope equipped with a video camera connected to a computer (Leica DM-RB, Hitachi HV-C20, PowerMacintosh 8100/80).

AnxV-biotin

AnxV-biotin (APOPTEST-BIOTIN®, product B500) was obtained from NeXins Research B.V. (Hoeven, The Netherlands). For control experiments the phospholipid binding property of AnxV-biotin was irreversibly destroyed by heating the protein for 10 minutes at 56°C (32).

Microinjection AnxV-biotin

Embryos were injected into the ventricle of the heart using a Hamilton-Syringe based pipette system using glass pipettes with a tip diameter of 15-25 µm. A volume of approximately 3 µl of the AnxV-biotin solution was injected under a surgical microscope while the embryo was kept in HEPES buffer of 37°C. When successfully injected, a temporary blanching of the umbilical vein could be seen. After 30 minutes of incubation, the embryos were examined for heart activity. Successfully injected embryos with positive heart activity were fixed and processed for visualization of bound AnxV-biotin.

AnxV-biotin; Whole Mounts

After overnight fixation in 4% Formalin/HEPES buffer at 4°C, embryos were washed in PBS and in 0.3% Triton X 100 in PBS, followed by digestion with 0.01% proteinase K (Boehringer-Mannheim, Mannheim, Germany) for 10 minutes. Endogenous peroxidase activity was blocked by incubation in 1% H_2O_2 in Tris/EDTA for 60 minutes. After washing with PBS, bound AnxV-biotin was visualized in the embryos at room temperature using the avidin-biotin complex method with horseradish peroxidase conjugated avidin (Vector ABC Elite kit, Brunschwig, Germany), with 3,3'-Diaminobenzidin tetrahydrochlorid (DAB) (0.05%) as a substrate. Specimens were stored in Tris/EDTA at 4°C until examination under a microscope.

AnxV-biotin; LM

Following overnight fixation in 4% Formalin/HEPES buffer at 4°C, embryos were dehydrated, embedded in paraffin, and serially sectioned at 3 µm. Endogenous peroxidase was blocked by incubation in methanol/H_2O_2 (9:1 v/v) for 20 minutes. Sections were washed in PBS and bound AnxV-biotin was visualized as described above. Sections were counterstained with Hematoxylin.

AnxV-biotin; EM

Day 13 embryos were microinjected with AnxV-biotin as described above and subsequently perfused intracardially with 0.5 ml 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. The limbs were removed, postfixed overnight in the same fixative, and cut on a Vibratome into 50 µm sections, which were processed to visualize the biotinylated Annexin V as described for LM. After the DAB reaction, the sections were subsequently postfixed in 1.5% OsO_4 in a 8% glucose solution, rinsed in destilled water, stained en bloc in 3% uranyl acetate, dehydrated in dimethoxypropane, and embedded in Dur-

cupan (8). Ultrathin sections were cut on an ultratome (Reichert Ultracut S, Leica, Germany), stained with leadcitrate, and examined under a Philips electron microscope (CM100).

Double Labeling With AnxV-biotin and TUNEL

Major DNA fragmentation as detected by in situ end-labelling procedures (27,49) has been described as a relatively late event in the process of apoptosis and is a marked feature of DNA containing phagocytosed apoptotic bodies (7). To evaluate whether cells with apoptotic morphology and fragmented DNA indeed showed AnxV positivity, sections stained for AnxV-biotin as described above were subjected to a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) procedure. Based on previous experience (21) we optimized the procedure in terms of signal-enhancement and tissue preservation: best results were obtained by preheating the specimens in 10 mM citrate buffer (pH 6.0) for 10 minutes at 55°C, followed by a slow cooling down to room temperature. Subsequently the sections were treated with 20 µg/ml DNase free proteinase K (Gibco, BRL Life Sciences, Merelbeke, Belgium) for 45 minutes at 37°C and washed 3 times for 5 minutes in Tris buffered saline (TBS). The labeling reaction was performed by incubating the sections under coverslides for one hour at 37°C with the following reaction mixture: 0.016 nmol/µl digoxygenin-11-2'-deoxyuridine-5'-tri-phosphate (DIG-11-dUTP, Boehringer Mannheim, Mannheim, Germany) and 0,135 U/µl calve-thymus terminal deoxynucleotidyl transferrase (TdT, Boehringer Mannheim) in a buffer containing 0,2 M cacodylate, 0,025 M Tris/HCL, 0,24 ng/ml bovine serum albumine, and 1 mM $CoCL_2$ (pH 6,6). After a preincubation step with block buffer (Boehringer Mannheim), specimens were incubated with alkaline phosphatase-conjugated Fab fragments from sheep raised against DIG (Boehringer Mannheim) for 1 hour. The reaction was developed with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT-PCIP) in Tris buffer (pH 9,5) for 5 minutes, giving a dark blue reaction product. After the staining for DNA fragmentation, the sections were mounted with aqueous mountant, without counterstaining.

RESULTS

Distribution of Intracardially Administered AnxV-biotin in the Viable Embryo

In vivo detection of cell surface exposed PS using AnxV-biotin requires the protein to be administered to the intact and living embryo by intracardiac injection. Annexin V is thus distributed by the specimen's own circulatory system and the protein can reach only the outer cell surface (Fig. 1). To verify whether the staining reflected specific binding that was dependent on the PS binding property of Annexin V, control embryos were injected with heat inactivated AnxV-biotin (32). This AnxV-biotin with a destroyed phospholipid binding activity did not bind to embryonic cells (Fig. 2); hence the observed staining with intact protein indicates specific interaction of AnxV-biotin with the plasma membranes.

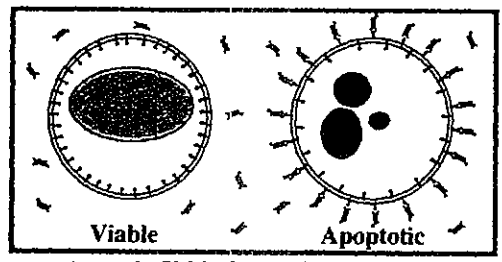

FIG. 1. A schematical representation of AnxV-biotin present in the interstitial compartment of viable mouse embryos after intracardial administration of the protein. In viable cells, the PS molecules are predominantly facing the cytoplasm and cannot be reached by AnxV-biotin. The surface exposure of PS by apoptotic cells provides binding sites for the AnxV-biotin molecules.

Annexin V Binding Cells Are Located in Regions of Developmental Cell Death

An accurate spatiotemporal correlation was found between AnxV-biotin binding patterns in the limbs and the apoptosis patterns known from literature and with those observed using the vital dye Nile blue sulphate (18,19,24,26,41,51) (Fig. 3). Despite the differences in morphology of the fore and hind limbs in day 11-13 embryos, the cell death patterns present were essentially similar. On 11 days of gestation, cells were labeled both in the apical ectodermal ridge (AER) and preaxially and postaxially in the underlying mesoderm (Fig. 3A1-A4). At day 12, labeled cells were still present in the AER, but their numbers were decreased compared to day 11 (Fig. 3B4). Intense labeling was present in the narrow band of mesodermal cells in between the AER and the marginal sinus. In this so-called progress zone, labeling was observed at the pre- and postaxial margins of the autopod in early day 12 embryos (Fig. 3B1 and 3B3-3B4), whereas in late day 12 embryos labeling was most prominently present in the distal parts of the interdigital areas (3B2) (18). Finally, at day 13, labeled cells were virtually absent from the AER, but present in large numbers preaxially, postaxially, and between the digits 1 and 5 (Fig. 3C1-3C4). Also at sites of joint formation in the stylopod, and in between the zeugopod and autopod, AnxV-biotin marked cells were present (Fig. 3C3 and 3C4).

Also at other sites where cell death is known to be present during embryogenesis (13), cells were AnxV-biotin labeled. Staining was distributed left-right symmetrically in the embryos and was present in cells derived from each germ layer. Examples are shown of ectoderm derived cells in the facial processes (Fig. 4A1-A2) and ganglia (Fig. 4B), of endoderm derived cells in the developing lung (Fig 4C), and of cells from mesodermal origin, i.e., somitic cells

Figure 2

Figure 4

Figure 3

FIGURES 2-4.

(Fig. 3A1-A2 and Fig. 4D) and cells of the wolffian (Fig. 4E) and müllerian ducts (Fig. 4F).

Occasionally, pyknotic cells were observed that were unlabeled for AnxV-biotin (see Fig. 4E). At the EM level, such unlabeled cells proved to be located within phagocytes. Since phagocytes containing AnxV-biotin positive cells were also present in the material (see below), the unlabeled cells presumably were phagocytosed before perfusion of the specimens with AnxV-biotin.

Early and Late Apoptotic Cells Bind Annexin V

For the EM study on the morphology of AnxV-biotin binding cells, we used sections through the interdigital mesodermal tissue from day 13 embryos. Some of the AnxV-biotin binding cells showed only subtle morphological differences with vital non-labeled cells. These labeled cells, which supposedly represent the early stages of apoptosis, showed signs of chromatin and cytoplasmatic condensation (Fig. 5A). An AnxV-biotin labeled cell with a rounded-off appearance, and more profound chromatin and cytoplasmatic condensation, is shown in Figure 5B; this cell is located in a phagocyte. The AnxV-biotin binding cells at later stages of apoptosis were clearly recognizable by their advanced chromatin condensation or by nuclear pyknosis (Fig. 5C). The surrounding viable cells did not show labelling at the plasma membrane.

The labeling for AnxV-biotin and TUNEL was revealed at the light microscopical level as a staining at the plasma membrane and nucleus, respectively. Cells in earlier stages of apoptosis were only AnxV-biotin positive (Fig. 6A1-A2), whereas cells showing both features were all in later stages of apoptosis, i.e., with profound chromatin condensation (Fig. 6A2-B). The pyknotic cell fragments were often only TUNEL-labeled (Fig. 6A1-A2).

DISCUSSION

The study of apoptotic cell death during embryogenesis not only assists in the understanding of normal development (13,25,26,28,44,45,48), but also aids in understanding of a variety of congenital malformations (14,15,18, 19,24,29,46,51). At present, apoptotic cells are detected mostly in the (serially sectioned) specimens on the basis of cell morphology, through the microscope, as it was done 150 years ago (5,13). Though widely applicable, this method is very time consuming, and at the LM level it permits the detection of only the condensed chromatin structure of late apoptotic cells. Alternatively, one may use vital dyes, like Nile blue sulfate (18,19,24,26,41,51), but the use of such dyes is restricted to visualization of cell death in whole mounts. In addition, biochemical markers for apoptosis like TUNEL (12) and ISEL are available (49). These markers detect apoptotic cells at the LM (25,49) and EM (30) level, but they can not be applied to whole mounts.

Studies of developmental cell death may benefit from early detection methods that are selective and can be applied to sections and whole mounts. The method for the in situ detection of apoptotic cell death that is presented in this study is based upon the injection of AnxV-biotin intracardially into viable mouse embryos. The apoptosis patterns in the limbs as visualized in whole specimens using the vital dye Nile blue sulphate (this study and 18,19,24,26,41, and 51) closely match with the AnxV-biotin staining in whole specimens and tissue sections. In subsequent stages of development, cell labeling was observed at various locations, e.g., in the AER, pre- and postaxially, where apoptosis serves the shaping of the hand/footplate; interdigitally, where it is required to separate the digits from each other; and in the presumptive joint-areas, where it is involved in cavity formation (26). AnxV-biotin labeling of apoptotic cells was not restricted to limb tissue, but was also present at other sites in the embryos. Staining was observed at sites in the embryo (1) where cell death serves fusion, e.g., in the facial processes (44,46); (2) where it occurs as degeneration of individual cells, e.g., neurons (22,38) and somitic cells (13); or (3) where it affects whole cell regions, e.g., wolffian duct and müllerian duct (13). In addition, it was observed at sites where the function of apoptosis is still enigmatic, e.g., the lung (13).

FIG. 2. Paraffin section through limb tissue of a heat inactivated-AnxV-biotin injected day 13 mouse embryo showing an absence of DAB staining in the interdigital degenerating tissue (Fig. A1; compare to Fig. 3C3-3C4). Figure A2 depicts a higher magnification of the boxed area in A1, showing apoptotic/pyknotic cells that are unlabeled for AnxV-biotin (arrows). Also, the embryonic erythroblasts were unlabeled (Fig. A1 and A3, arrowheads). Scale bars equal 200 μm (A1) or 25 μm (A2, A3).

FIG. 3. Apoptosis patterns in limbs from day 11 (A), day 12 (B), and day 13 (C) mouse embryos, visualized in whole limbs using Nile blue sulfate as a marker (A1-C1), and in whole mounts (A2-C2) and paraffin sections (A3-C3 and Ai-Ci) using Annexin V. Within one age group, boxed areas represent similar regions in Nile blue sulfate treated embryos and in Annexin V treated embryos. In Ai-Ci higher magnifications are shown of the areas boxed in A1-C1, A2-C2, and A3-C3. At day 11, cells were marked in the limbs in both the AER (A3-Ai: arrowhead) and pre- and postaxially in the underlying mesoderm (Ai: arrow). Notice the Nile blue sulfate and AnxV-biotin stained somites (A1-A2: arrow). At day 12, labeling was observed in the narrow band of mesodermal cells in between the marginal sinus (Bi: MS) and the AER, i.e., the progress zone (Bi: arrow). This labeling was present at the pre- and postaxial margins of the autopods of early day 12 embryos (B1 and B3-Bi), whereas it has shifted to the distal interdigital regions in late day 12 embryos (B2). Also, marked cells were still present in the AER but in decreased numbers with respect to day 11 (Bi: arrowhead). In day 13 mouse embryos, labeled cells were virtually absent in the AER (Ci: arrowhead), but present in large numbers pre- and postaxially and in between the digits 1-5 (C1-C3: boxed areas, and Ci: arrow). Also, at sites of joint formation in the stylopod, Annexin V positive cells were found (Ci: asterix). Scale bars equal 20 μm (Ai, Bi), 100 μm (Ci), or 500 μm (A1-C1, A2-C2, and A3-C3).

FIG. 4. General presence of Annexin V labeling in paraffin embedded serially sectioned day 11 (A,C,D), day 12 (B) and day 13 (E,F) mouse embryos. In A1, sagittal section showing fusion of the lateral nasal and maxillary processes. In A2, a higher magnification is shown of the boxed area in A1, depicting AnxV-biotin labeled cells (arrow) in the center of fusion of the facial processes. (B) Section through the trigeminal ganglion showing labeled cells with a pyknotic and often also fragmented nucleus (arrow). (C) Labeled cells in the endoderm derived bronchial epithelium (arrows). In transverse sections through the tail region cells were observed labeled in the degenerating tailgut and in the somites at site of the presumptive somitocoel (D, arrow). Annexin V labeled cells were also abundantly present in the urogenital system. Examples are shown of the wolffian (E) and müllerian ducts (F), in the region where the ducts are bound to orifice into the urogenital sinus (asterix). Scale bars equal 10 μm (B,D), 25 μm (A2, C, E, and F), or 100 μm (A1).

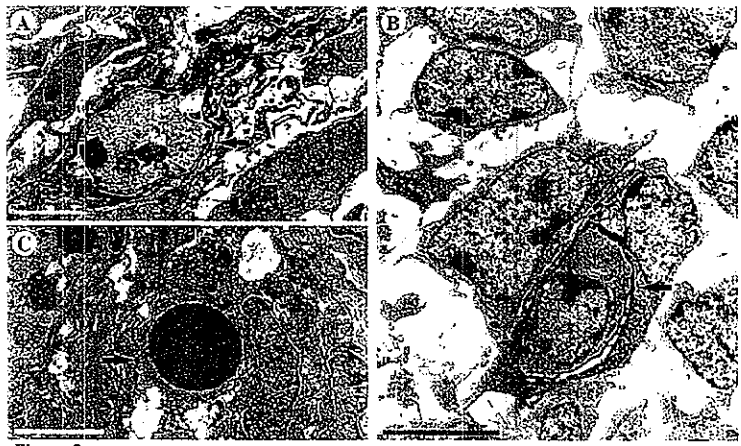

Figure 5

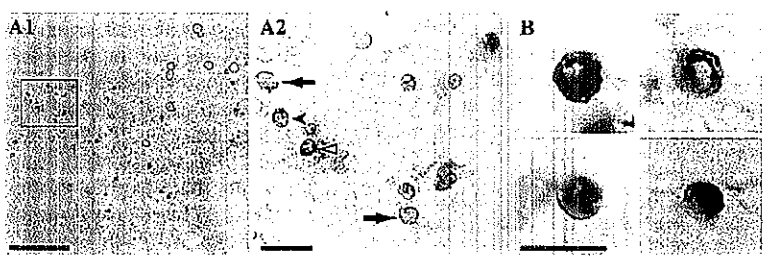

Figure 6

FIG. 5. Electron micrographs, showing AnxV-biotin labeled cells located in the interdigital region of a day 13 mouse embryo. Cells were found labeled at early stages of apoptosis, when showing first signs of chromatin condensation (A), ingested by a phagocyte (B), and up to the later stages of apoptosis when clearly pyknotic (C). Viable cells were unlabeled (asterix). Scale bar equals 2 μm.

FIG. 6. Paraffin sections through day 13 limbs of AnxV-biotin injected mouse embryos that have been stained both for PS exposure at the plasma membrane via AnxV-biotin and for DNA fragmentation via the TUNEL method; no counterstaining was applied. In figure A2 a higher magnification is shown of the boxed area in figure A1, showing in one field the three types of labeled cells that were observed: (i) cells that showed AnxV-biotin-labeling at the plasma membrane only (arrows), (ii) pyknotic cells that were double labeled for AnxV-biotin and TUNEL (arrowhead), and (iii) pyknotic cells and cell fragments that appeared only TUNEL-positive (open arrowhead). In B, four AnxV+/TUNEL+ cells are depicted in subsequent stages of apoptosis. Such cells were having chromatin margination (upper left and upper right) or nuclear fragmentation (lower left), or were clearly pyknotic (lower right). Scale bar equals 50 μm (A1) or 10 μm (A2) or 5 μm (B).

AnxV-biotin can also be used to detect apoptotic cells at the ultrastructural level. These cells were characterized by their typical morphology (50); their appearance ranged from early stages of apoptosis, where the cells show the first signs of cytoplasmatic and nuclear changes to the later stages, with advanced cytoplasmatic and chromatin condensation and ultimate pyknosis. Additional evidence for the association between apoptosis and PS exposure at the outer layer of the plasma membrane came from double labeling experiments, applying the TUNEL protocol for detection of apoptosis associated internucleosomal DNA fragmentation (12,21) subsequent to the Annexin V protocol. The double labeled cells were all in the late, pyknotic phase of apoptosis. In contrast, the apoptotic cells in relatively early stages of apoptosis were only AnxV-biotin labeled at the plasma membrane. The presence of these AnxV-biotin-positive and TUNEL negative cells suggests that loss of PS plasma membrane asymmetry precedes the stages when DNA fragmentation can be detected by TUNEL. A similar sequence of events was observed for hematopoietic cells entering apoptosis in vitro (4,23). The double labelling experiments also showed that some of the pyknotic cells and cell fragments were only TUNEL positive. At the EM level, the pyknotic cells without AnxV-biotin labeling at the plasma membrane were shown to be located within phagocytes. We hypothesize that these apoptotic cells were already ingested by phagocytes before the embryos were injected with the AnxV-biotin, and therefore could not be reached by the circulating protein.

In conclusion, we demonstrated the feasibility of Annexin V in vivo labeling of apoptotic cells in whole mounts and tissue sections of mouse embryos at the light- and ultrastructural level.

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Spatiotemporal distribution of dying neurons during early mouse development

S. M. van den Eijnde,[1,2*] J. Lips,[3*] L. Boshart,[1] C. Vermeij-Keers,[2,3] E. Marani,[5] C. P. M. Reutelingsperger,[4] and C. I. De Zeeuw[3]

[1]MGC Department of Clinical Genetics, Erasmus University Medical School, PO Box 1738, 3000DR Rotterdam, the Netherlands
[2]Institute of Plastic Surgery, Erasmus University Medical School, PO Box 1738, 3000DR Rotterdam, the Netherlands
[3]Department of Anatomy, Erasmus University Medical School, PO Box 1738, 3000DR Rotterdam, the Netherlands
[4]Department of Biochemistry, Cardiovascular Research Institute Maastricht, University of Maastricht, Universiteitssingel 50, 6229ER Maastricht, the Netherlands
[5]Department of Physiology, Leiden State University Medical School, PO Box 9602, 2300 RC Leiden, the Netherlands

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Abstract

Apoptosis is a critical cellular event during several stages of neuronal development. Recently, we have shown that biotinylated annexin V detects apoptosis *in vivo* in various cell lineages of a wide range of species by binding to phosphatidylserines that are exposed at the outer leaflet of the plasma membrane. In the present study, we tested the specificity by which annexin V binds apoptotic neurons, and subsequently investigated developmental cell death in the central and peripheral nervous system of early mouse embryos at both the cellular and histological level, and compared the phagocytic clearance of apoptotic neurons with that of apoptotic mesodermal cells. Our data indicate: (i) that biotinylated annexin V can be used as a sensitive marker that detects apoptotic neurons, including their extensions at an early stage during development; (ii) that apoptosis plays an important part during early morphogenesis of the central nervous system, and during early quantitative matching of brain-derived neurotrophic factor and neurotrophic factor 3 responsive postmitotic large clear neurons in the peripheral ganglia with their projection areas; and (iii) that apoptotic neurons are removed by a process that differs from classical phagocytosis of non-neuronal tissues.

Introduction

Apoptosis is abundant in the developing central (CNS) and peripheral (PNS) nervous system (Oppenheim, 1991; Blaschke *et al.*, 1995), and may account for up to 50–70% of the loss of neurons (Cowan *et al.*, 1984; Clarke, 1985). The number of surviving neurons appears to be controlled by their afferent input and by the presence of trophic factors in peripheral target areas (Davies, 1987; Barde, 1989; Linden, 1994). When such survival factors are lacking during critical times in development, the apoptosis programme is initiated and the neuronal cell bodies and neurites start to disintegrate (Martin *et al.*, 1988; Schwartz *et al.*, 1993; Estus *et al.*, 1994; Jackobson *et al.*, 1994; Reed, 1994; Mesner *et al.*, 1995). Typical morphological characteristics of the apoptotic cells are chromatin condensation and pyknosis, volume reduction, freeing of ribosomes from polyribosomes, altered electron density of the cytoplasm, membrane blebbing and fragmentation into apoptotic bodies (Kerr *et al.*, 1972; Wyllie *et al.*, 1980).

In both neuronal and non-neuronal tissues, the removal of apoptotic cells by adjacent cells or macrophages takes place in a relatively short period without invoking an inflammatory response from the surrounding tissue as occurs during necrosis (Wyllie *et al.*, 1980; Ferrer *et al.*, 1990; Fadok *et al.*, 1998; Savill, 1998). This removal requires specific surface elements to be present on the dying cells, enabling binding to and engulfment by phagocytes. While specific epitopes have been described to exist on apoptotic cells during development (Ellis *et al.*, 1991; Rotello *et al.*, 1994), the only functionality of such factors in the process of phagocytic clearance has been revealed in *in vitro* experiments of 'adult' cells in culture (Savill *et al.*, 1993; Ren *et al.*, 1995). One of these factors is the aminophospholipid phosphatidylserine (PS) (Fadok *et al.*, 1992b; Fadok *et al.*, 1993).

In viable cells PSs predominantly reside in the inner leaflet of the plasma membrane bilayer through the activity of an energy consuming aminophospholipid translocase (Diaz & Schroit, 1996; Zwaal & Schroit, 1997). During apoptosis, the PS molecules become exposed at the outer plasma membrane leaflet after inhibition of the translocase and activation of a scramblase (Higgins, 1994; Verhoven *et al.*, 1995). Annexin V, which binds PS in a Ca^{2+}-dependent manner (Tait *et al.*, 1989; Andree *et al.*, 1990), has been found to be a reliable marker for apoptosis both *in vitro* and *in vivo* (Koopman *et al.*, 1994; Martin *et al.*, 1995; van den Eijnde *et al.*, 1997a,b; 1998).

In the present study we show that annexin V linked to a fluorescent label or biotin specifically binds cell bodies and extensions of early (non-pyknotic) and late (pyknotic) apoptotic neurons both *in vitro* and *in vivo*, respectively. Using this marker, we explored apoptosis in the CNS and PNS in early mouse embryos [embryonic day (E)

Correspondence: Stefan M. van den Eijnde, Institute of Plastic Surgery, Erasmus University Medical School, PO Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: vandeneijnde@ikg.fgg.eur.nl. Also Chris I. De Zeeuw, as above. E-mail: dezeeuw@anat.fgg.eur.nl

*These authors contributed equally to this study

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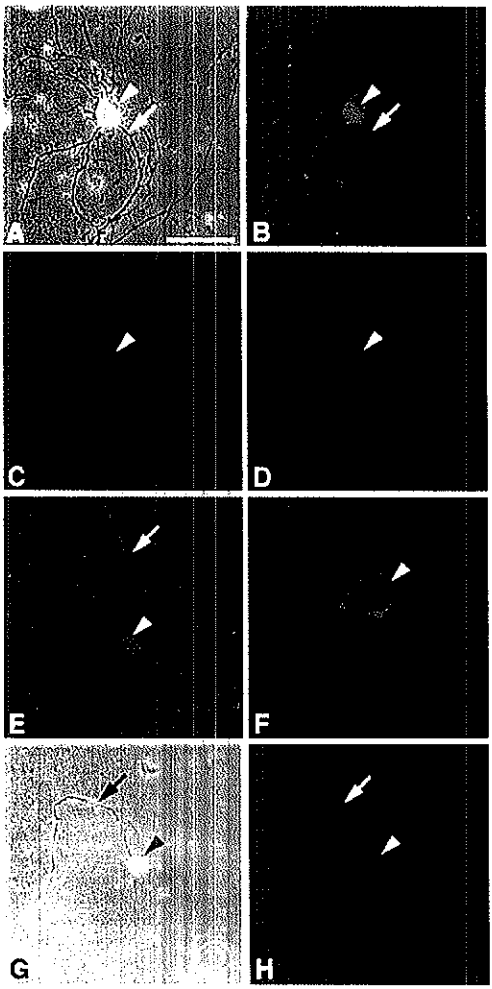


FIG. 1. AnxV-OG labels apoptotic DRG neurons *in vitro* following NGF withdrawal. (A–F) Show cultured DRG neurons after 6 h of NGF deprivation with the use of phase contrast microscopy (A), fluorescent annexin V labelling, nuclear Hoechst 33342 staining, and PI staining. (A–D) Show an early apoptotic neuron, which as revealed by the annexin V labelling, exposes its phosphatidylserines at both the soma (B: arrowhead) and neurites (B: arrow). The cell's pyknotic nucleus is stained with Hoechst 33342 (C: arrowhead), whereas the lack of PI staining indicates that the plasma membrane integrity is intact (D: arrowhead). Some annexin V positive and propidium negative cells were characterized by a more intense staining of the nucleus with Hoechst 33342 staining, and were probably in later phases of apoptosis (E and F; triple staining). G (phase contrast) and F (triple fluorescent staining with annexin V, Hoechst 33342, and PI) show the absence of apoptosis of a DRG neuron cultured (soma: arrowhead; neurite: arrow) in the presence and absence of NGF and anti-NGF, respectively. Scale bar equals 25 μm, except for figure F, where scale bar equals 10 μm.

9–14]. Instead of providing an in-depth overview of an isolated aspect of neuronal cell death, we present this issue from a wide range of perspectives, varying from monitoring cell death at the cellular level to evaluating the general spatiotemporal distribution and phagocytosis of neuronal apoptosis. Our results indicate that apoptosis in the CNS is linked with morphogenetic events, whereas in the PNS, the spatiotemporal cell death patterns direct towards a process of early matching of the phenotypically characteristic large clear neurons in the peripheral ganglia with their afferent and efferent target areas. In addition, we show that phagocytosis in neuronal tissue differs from phagocytosis in non-neuronal tissues.

Materials and methods

Annexin V labelling of nerve growth factor-deprived apoptotic dorsal root ganglion cells in vitro

Neonatal Wistar rats aged P0–4 (i.e. postnatal days 0–4) were anaesthetized by cooling at 4 °C for 20 min, and subsequently decapitated and rinsed in 70% ethanol and sterile saline. The dorsal part of the vertebral column was cut open in the caudorostral direction and the dorsal root ganglia (DRGs) with attached nerve stumps and spinal roots were removed aseptically. The DRG cells were dissociated mechanically, and processed and maintained on poly-D-lysine-coated cover slips in chemically defined medium (R12), which was changed three times a week (Romijn *et al.*, 1984; van Dorp *et al.*, 1990). On days 4–7 NGF-2.5S (Sigma, Bornhem, Belgium) was added to the DRG cultures at a final concentration of 1 μg/mL.

After 7–10 days the neurons had formed stable connections (Fig. 1A) and NGF was removed from the dissociated DRG cultures ($n = 35$) by adding medium with anti-NGF-2.5S (Sigma, Bornhem, Belgium) at a final concentration of 1 : 500 (Martin *et al.*, 1988). After 6–27 h of incubation with anti-NGF the supernatant was removed, the cells were rinsed with HEPES buffer [20 mM HEPES (pH 7.4), 132 mM NaCl, 2.5 mM $CaCl_2$, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, 0.5% bovine serum albumin], and resuspended in chemical defined medium (R12: Romijn *et al.*, 1984) containing 250 ng/mL of annexin V conjugated to Oregon Green (AnxV-OG: Apoptest- Annexin V-OregonGreen, G-700, NeXins Research BV, Hoeven, The Netherlands). Also, to each culture bisbenzimide (Hoechst 33342; final concentration 10 μg/mL) (Sigma, Bornhem, Belgium) and propidium iodide (PI; final concentration 5 μg/mL) were added for evaluation of nuclear morphology and membrane integrity, respectively (Lizard *et al.*, 1995). The cells were maintained for 20 min at 4 °C and subsequently fixed for another 20 min in 4% formalin in HEPES buffer, rinsed in HEPES buffer and mounted with UV inert aqueous mountant (Gurr, High Wycombe, Bucks, UK). Controls ($n = 5$) were cultured for 7–10 days in NGF-containing medium without the addition of anti-NGF. Light microscopic analysis of the cultured neurons was performed with a fluorescent microscope (Leica DM-RB, Wetzlar, Germany) with standard filters for Oregon Green (λ Ex 488 nm; λ Em 520 nm), Hoechst 33342 (λ Ex 346 nm; λ Em 460 nm) and PI (λ Ex 540 nm; λ Em 625 nm). The morphological characteristics of the cell bodies and their neurites were assessed with phase-contrast microscopy.

Annexin V labelling during embryogenesis

Electron microscopy

E12 ($n = 6$) and E13 ($n = 6$) FVB mouse embryos (E0 = morning on which vaginal plug was found) were processed for detection of biotin conjugated annexin V (AnxV-biotin; APOPTEST-biotin, B-500, NeXins Research BV) at the electron microscopic level according to

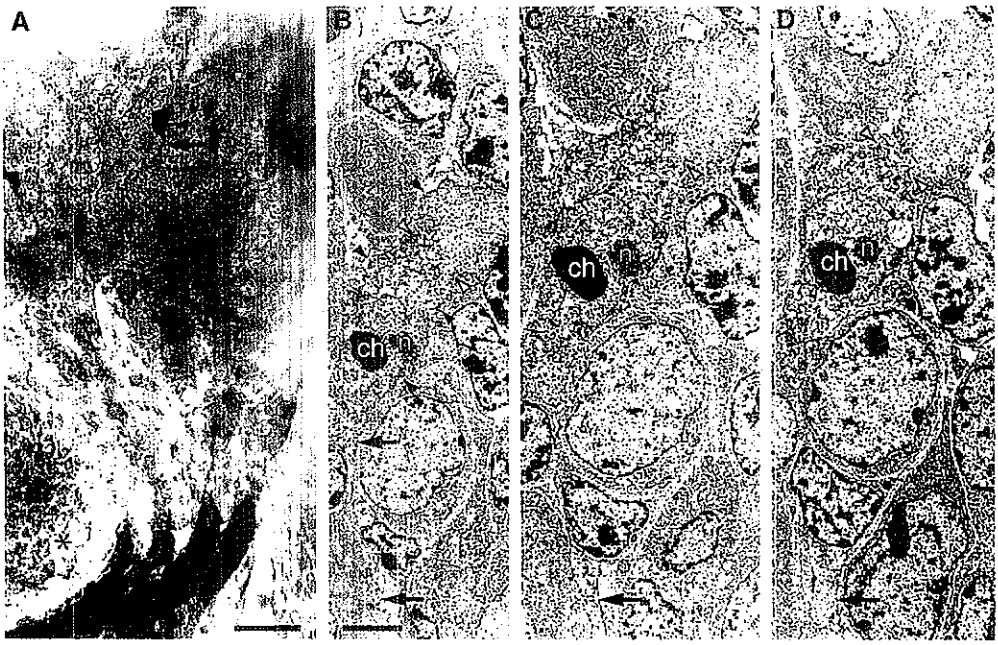


FIG. 2. Electron microscopic distribution of apoptotic neurons and their extensions in lumbal DRGs at E13. A shows the localization of an apoptotic neuron in a 50 µm-thick Vibratome section. Note the prominent AnxV-biotin labelling in both the soma and proximal parts of the extensions presented (asterix: spinal column). The ultrastructural, serial section analysis of this annexin V labelled apoptotic pseudo-unipolar neuron of the large/clear group shows that the entire plasma membrane of the cell is intact and labelled (B–D: closed arrowheads). The apoptotic morphology of the neuron can be recognized by the condensation of its chromatin (ch) and the fragmentation of its soma (open arrowheads). Note that the nucleus of the cell is not condensed and delineated by a dissolving nuclear membrane; the nucleolus (*n*) is still visible. The central projection of the neuron is located in the lower left quadrant (arrow). Sections are 400 nm apart. Scale bar indicates A, 17 µm; B–D, 2.3 µm.

procedures previously described (van den Eijnde *et al.*, 1997a,b). The embryos were dissected out of the uterus, and a volume of 3 µL of AnxV-biotin was injected through a glass micropipette (tip diameter ≈ 20 µm) into the ventricle of their heart. During this perfusion the embryo was kept in HEPES buffer at 37 °C. Successfully injected embryos that were alive after 30 min, were intracardially perfused with 0.5 mL 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. Embryos were postfixed overnight in the same fixative, and cut on a Vibratome (TPI Technical Products Int. Inc., St Louis, MO, USA) into 50 µm sections. Endogenous peroxidase activity was blocked by incubating the sections with 1% H_2O_2 in methanol for 20 min. After washing the sections with phosphate-buffered saline (PBS), bound AnxV-biotin was visualized using the avidin–biotin complex method with horseradish peroxidase conjugated avidin (ABC Elite kit, Vector Laboratories Inc., Burlingame, CA, USA). Staining was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.05% w/v) and 0.1% H_2O_2 in PBS; to intensify the staining 0.02% $CoCl_2$ was added to the DAB solution. After the reaction with DAB-Co, the sections were postfixed in 1.5% OsO_4 in a 8% glucose solution, rinsed in aquadest, stained *en bloc* in 3% uranyl acetate, dehydrated in dimethoxypropane and embedded in Durcupan (for details of EM procedures see: De Zeeuw *et al.*, 1988, 1989). Semithin and ultrathin tissue sections were cut on an ultratome (Ultracut S, Reichert Jung, Rijswijk, The Netherlands) and counterstained with Toluidine blue, and uranyl acetate and lead citrate, respectively. The ultrathin sections were examined under a Philips electron microscope (CM 100, Eindhoven, the Netherlands). For control, two E12 and two E13 FVB mouse embryos were processed as described above, except that they received an injection with HEPES buffer instead of biotinylated annexin V.

Light microscopy

AnxV-biotin was injected into the heart of E9–13 mouse embryos, and/or the sinus sigmoideus in the head of E13–14 embryos. At least six embryos from each developmental age, derived from two or more dams, were injected with the protein. After 30 min of survival, the embryos were fixed in ice-cold HEPES buffer containing 4% formaldehyde, dehydrated in a graded series of ethanol, incubated with toluol, toluol/paraffin (1 : 1 v/v), embedded in paraffin and serially sectioned at 3 µm in a sagittal or transverse plane. The procedures for visualization of bound AnxV-biotin were as described for electron microscopy, except that the DAB reaction was not intensified with $CoCl_2$. The sections were rinsed with water, counterstained with Gills Haematoxylin (GHS-2, Sigma, Bornhem, Belgium) and mounted. To test for non-specific binding of annexin V, embryos were injected with AnxV-biotin that was heat inactivated

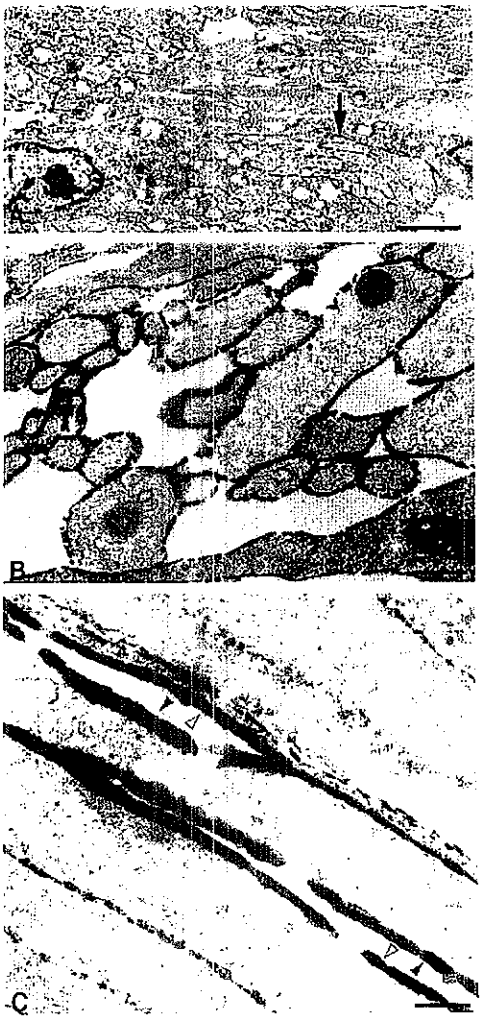


FIG. 3. Ultrastructure of annexin V labelled peripheral axons from lumbal DRG neurons at E13. A (arrows) and B show that the labelled axons can occur in clusters. (C) Shows that the AnxV-biotin labelling is not restricted to the membranes of the apoptotic axon itself (closed arrowheads), but that it extends to the membranes of the neighbouring axons (open arrowheads). Scale bars in A, B and C indicate 4.7 μm, 0.6 μm, and 0.4 μm, respectively.

for 10 min at 56 °C (n = 16; van den Eijnde *et al.*, 1997a); in none of these control embryos AnxV-biotin labelling was observed.

Quantification

In serial sagittal paraffin-sections through E11–13 embryos (n = 8) AnxV-biotin labelled neurons were counted bilaterally in all DRGs present along the rostrocaudal axis as well as in the trigeminal ganglia. The first DRG was determined by its relative orientation to the anlagen of the occipital and the atlas bones. AnxV-biotin positive neurons with a clear nuclear profile (maximum diameter ≈ 9 μm) were counted only in each third section, thus limiting double counting of neurons. Of each of these sections, the percentage of labelled cells per total cell number was determined (Thomaidou *et al.*, 1997). In addition, we determined the percentage of unlabelled pyknotic cells in the DRGs at levels Th6–8 and in the trigeminal ganglia. The numbers were averaged and the standard errors of the mean (SEM) were calculated for the different ganglia and embryonic stages. Statistical analysis of these data was performed with Student's t-test using Sigma Stat software (Jandel Scientific, Chicago, IL, USA).

Results

Annexin V binds apoptotic neuronal cell bodies and neurites

Neuronal apoptosis induced in vitro

NGF deprivation is a well established method to induce neuronal cell death (Martin *et al.*, 1988; Barde, 1989; Mesner *et al.*, 1995). Here, we induced apoptosis in dissociated neonatal DRG neurons by withdrawing NGF, and we detected PS exposure of these apoptotic cells by incubating them with AnxV-OG. Six hours after NGF withdrawal, the first apoptotic neurons and their neurites bound annexin V. Ten to 15 h following NGF withdrawal, the plasma membrane of both the soma and neurites became intensely annexin V positive (Fig. 1B,E,F). Such cells routinely exhibited limited (Fig. 1C) to extensive (Fig. 1E,F) nuclear pyknosis and intact plasma membranes as revealed by positive Hoechst 33342 staining and an absence of PI labelling (Fig. 1D–F), respectively. In control experiments (Fig. 1G,H), in which cells were kept continuously in NGF-containing medium, little or no labelling with annexin V or PI was present, and virtually all nuclei had an uncondensed 'vital' morphology (Fig. 1H). Whereas annexin V unlabelled pyknotic cells were generally not observed, in all cultures both with and without anti-NGF a few neurons were usually labelled with both annexin V and PI, indicating a compromised integrity of the plasma membrane. These cells showed a discontinuous plasma membrane-labelling with AnxV-OG in both the perikaryon and the disrupted neurites, and were probably post-apoptotic- or secondary necrotic, as suggested by the pyknotic and often fragmented nucleus (Darzynkiewicz *et al.*, 1997). Thus, annexin V binds specifically both the soma and neurites of apoptotic neurons when the plasma membrane integrity is preserved, but if one studies a preparation with abundant neuronal necrosis, assessment of the integrity of the plasma membrane with PI is critical to discriminate both forms of cell death.

Ultrastructural characterization of neuronal apoptosis in vivo

To find out whether annexin V also labels apoptotic neurons *in vivo*, we investigated lumbar DRGs and trigeminal ganglion neurons of E12 and E13 embryos at the ultrastructural level following intracardial injection of AnxV-biotin. Many positively labelled neurons exhibited the morphological characteristics that are typical of late apoptosis such as a pyknotic nucleus with condensed chromatin and cell fragmentation. Other labelled cells showed morphological features that are not typical of late apoptosis, but still compatible with this process or with earlier forms of it. These included a dissolving nuclear membrane, a dilated endoplasmatic reticulum, membrane blebbing, and an increased number of free ribosomes and a reduced electron density of the cytoplasm (Fig. 2). In contrast to the usual

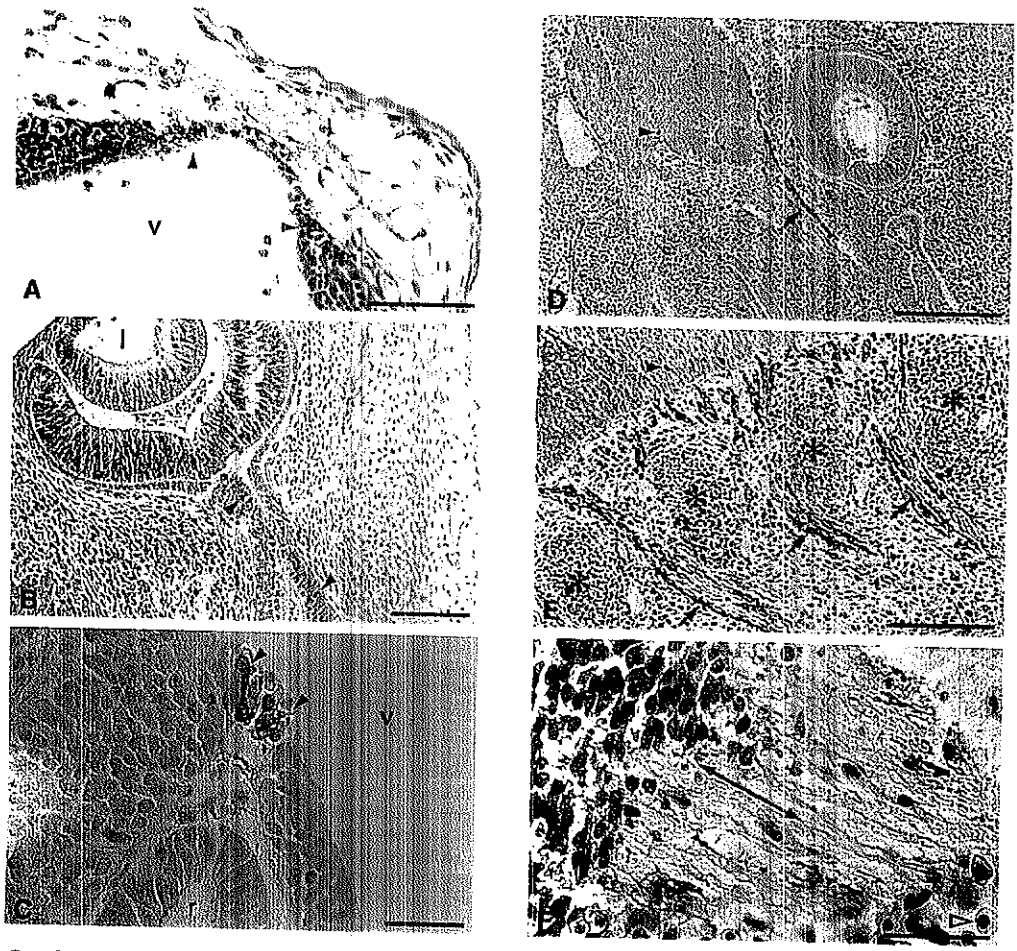


FIG. 4. Low power micrographs of paraffin sections showing AnxV-biotin labelling of cells and extensions in the CNS (A–C) and PNS (D–F). With respect to the CNS, examples of clusters of labelled neurons (arrowheads) are illustrated for the laterorostral border of the cerebellum (A; E12), the optic stalk and fissure (B; E11), and Rathke's pouch (C; E11). With respect to the PNS, examples of clusters of labelled extensions (arrows) are illustrated for the trigeminal ganglion and its maxillar branch (D; E11), spinal nerves emerging between the vertebrae (asterisks) (E; E11), and fibres from the cauda equina (F; E12). Note in A that the area with clusters of labelled neurons of the cerebellar anlage is restricted to the thin edge of the wall of the rhombencephalon surrounding the fourth ventricle (v); the directly adjacent cylindrical epithelium of the rhombencephalon is merely devoid of apoptotic cells. L and r in B and C indicate lens vesicle and lumen of Rathke's pouch, respectively. The dark arrowheads in D and E indicate isolated annexin V labelled neurons, while the open arrowhead in F indicates an embryonic red blood cell. The double-headed arrow in F is orientated along the rostrocaudal axis. Scale bars in A–F indicate 19 μm, 100 μm, 25 μm, 33 μm, 25 μm and 28 μm, respectively.

appearance of the mitochondria in necrotic cells, those in labelled apoptotic neurons were often clustered and undilated. Interestingly, the cascade of apoptotic events in the cytoplasm did not always occur in parallel with those in the nuclei; frequently, we observed neurons with pyknotic nuclei characteristic of late apoptosis in conjunction with cytoplasmic changes characteristic of early forms of apoptosis and vice versa. The annexin V labelling in the apoptotic neurons was not restricted to their cell bodies, but also extended into their corresponding axons. The labelled axons in the peripheral nerves tended to be grouped in clusters (Fig. 3A,B). In these clusters, some axons of presumptive viable cells were labelled at the plasma membrane directly adjacent to the apoptotic neurites that were labelled across their entire perimeter, whereas the other parts of the axon were not labelled (Fig. 3C). A similar phenomenon was observed for the

labelled somata in the ganglia. These ultrastructural data indicate: (i) that annexin V labels the membranes of apoptotic neuronal somata and axons during different stages across their full perimeter; and (ii) that the membranes of viable cells can be labelled at the part that is directly adjacent to apoptotic neurons.

Light microscopic distribution of apoptotic neurons in the developing central and peripheral nervous system

Cytology

The ultrastructural characteristics of the cytological features of annexin V labelled neurons in the lumbar DRGs described above were extensively confirmed in our light microscopic examination of many different areas in the brains of E9–14 embryos (see Fig. 4). Many of the annexin V labelled neurons showed chromatin condensation, and depending on the extent of this feature these neurons could be characterized as early (Fig. 5A–C) or late (Fig. 5D) apoptotic. Frequently, both the neuronal soma and extensions of the cells were labelled, and often, it was possible to identify the regular shape of the apoptotic neuron. For example, the early apoptotic neurons from the thalamus and ventral horn of the spinal cord illustrated in Fig. 5A,B can be identified as bipolar and multipolar, respectively. In some cases, it was even possible to follow the peripheral axon to the soma of the apoptotic neuron in a single section (Fig. 5D). In the PNS both the early and late labelled apoptotic neurons belonged to the category of large clear cells, which are characterized by a large pale nucleus and cell body with a diameter of 19 µm (Lawson & Biscoe, 1979). Small dark cells (on average 12 µm in diameter), which may be predominantly derived from the neural crest (Davies & Lindsay, 1984), were never found to be labelled. In contrast to the apoptotic neurons in the CNS and PNS, embryonic red blood cells, which also show a condensed nucleus and resemble thereby late apoptotic cells with a pyknotic nucleus, did not bind annexin V to their plasma membrane (Figs 4F and 5D). Thus, the cytological observations at the light microscopic level *in vivo* agree with the findings of the *in vitro* experiments and electron microscopic study in that the use of biotinylated annexin V allows the identification of both early and late apoptotic neurons, and that it labels not only their cell bodies but also their dendrites and axons.

Central nervous system

In the CNS, apoptotic neurons were either grouped in clusters or dispersed throughout the neuroepithelium. In general, the clusters of apoptotic cells were prominently present during E11 and E12, but virtually absent at E9 and E14. They occurred at several anatomical landmarks associated with morphogenetic transformations of the neural tube. The four most pronounced sites in this respect included the laterorostral border of the thin wall of the cerebellar anlage in the rhombencephalon (Fig. 4A), the optic stalk including the optic fissure (Fig. 4B), the anlage of the posterior pituitary gland overlaying Rathke's pouch, i.e. the infundibulum of the diencephalon (Fig. 4C), and the velum transversum and plexus choroideus anlage in the wall of the third ventricle (Fig. 6). Chronological analyses of neuronal apoptosis in each of these sites indicated that most of the apoptosis occurs at specific sites of the curvatures in the neuroepithelium during a particular time frame. In the velum transversum for example, the concave parts of the lateral curvature appeared to be a constant site of apoptosis from E10 to E12 (for chronological series from E10 to E14, see Fig. 6). In contrast to the specific spatiotemporal patterns of apoptotic clusters, the dispersed cell death occurred rather randomly throughout the neural tube (Fig. 5A). The major exception was formed by the rostral parts of the telencephalic hemispheres, at which a relatively high level of this form of cell death was observed (data not shown). Different from the clustered cell death, the dispersed cell death persisted up to E14.

Peripheral nervous system

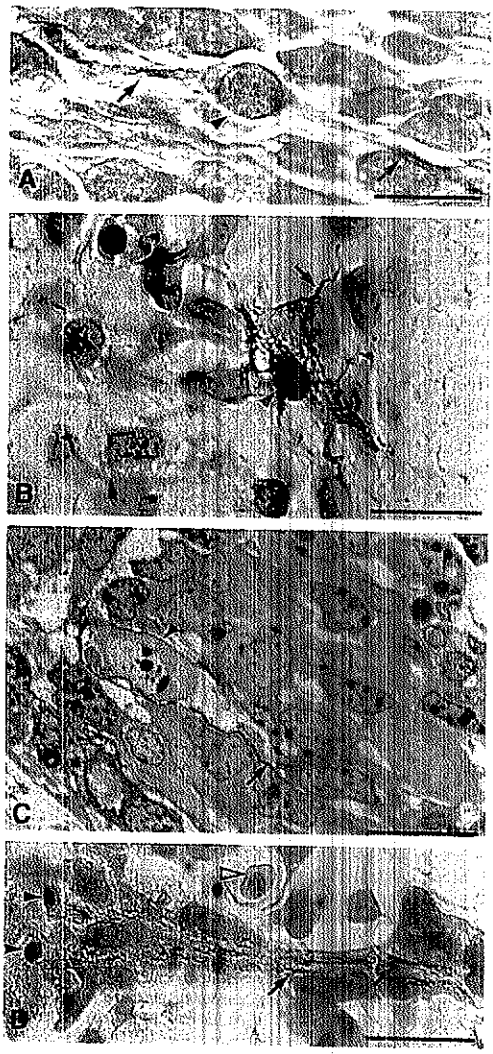
As observed in the CNS, neuronal apoptosis in the PNS was most abundantly present from E11 to E13. At E14, annexin V labelling was still observed in some of the cranial ganglia such as the ganglion of the trigeminal nerve, but the DRGs were almost completely devoid of apoptotic neurons. In contrast to the spatial labelling patterns in the CNS, apoptotic neurons in the PNS were not grouped in clusters but always dispersed throughout the neuropil. This labelling pattern held true for the ganglia of both the cranial nerves and spinal nerves. The labelling of the axons in the PNS was much more prominent than that in the CNS. Figures 4D–F illustrate, for example, the dense annexin V labelling in the second (maxillar) branch of the trigeminal nerve, spinal nerves and cauda equina, respectively. Interestingly, the onset of labelling of the axons was similar to that of the somata, but the labelling persisted up to 1 day longer in the axons than in the somata.

Quantification of the percentage of apoptotic cell bodies in the different DRGs from E11 to E13 revealed two features (Fig. 7A). First, the highest density of dying cells shifted from cranial to caudal DRGs during E11–13. At E11, the average percentage [0.95% ± 0.17 (SEM)] of annexin V labelled neurons in the rostral DRGs (C1–Th8) was significantly higher ($P < 0.001$; *t*-test) than that (0.27% ± 0.13) in the caudal DRGs (Th9–S4). At E12 and E13, however, the average percentages (0.39 ± 0.03 at E12 and 0.23 ± 0.03 at E13) of annexin V labelled neurons in the rostral DRGs were significantly lower ($P < 0.01$; *t*-test) than those (0.58 ± 0.05 at E12 and 0.36 ± 0.07 at E13) in the caudal DRGs. Second, the average percentage of annexin V labelled cell bodies of all DRGs decreased significantly over time from 0.58% [± 0.15 (SEM)] at E11 to 0.43% (± 0.04) and 0.27% (± 0.05) at E12 and E13, respectively ($P < 0.001$ for both E11 vs. E12 and E12 vs. E13; *t*-test). Thus, cell death in the PNS occurs in a particular spatiotemporal pattern along the rostrocaudal axis.

Relation between annexin V labelling and pyknosis

To assess the relative proportions of early vs. late apoptotic neurons in the nervous system, and to estimate the relative sensitivity of the annexin V labelling method for detecting apoptotic neurons, we determined the proportions of three different populations of apoptotic neurons in DRGs at levels Th6–8 as well as in trigeminal ganglia from E11 to E13 (Fig. 7B,C). These populations included: (i) annexin V-labelled, early apoptotic neurons with an uncondensed nucleus; (ii) annexin V-labelled, late apoptotic neurons with a pyknotic nucleus; and (iii) unlabelled neurons with a pyknotic nucleus.

In line with the time shift of the number of annexin V labelled neurons of all DRGs described above (Fig. 7A), the total number of apoptotic cells (i.e. the total of the three populations) in DRGs Th6–8 decreased over time from E11 to E13 (Fig. 7B). The proportions of the three populations remained relatively unchanged during this period. At E11, E12, as well as E13 ≈ 10% of the apoptotic cells in DRGs Th6–8 were annexin positive and early apoptotic, 40% of the cells were labelled and late apoptotic, and about half of the total apoptotic population showed advanced chromatin condensation but was unlabelled with annexin V. In the trigeminal ganglion, similar proportions were observed at these 3 days, but the highest level of apoptosis occurred at E12 instead of E11 (Fig. 7C). We can conclude that probably about half of all apoptotic neurons can be detected with biotinylated annexin V, and that, as far as the early apoptotic cells



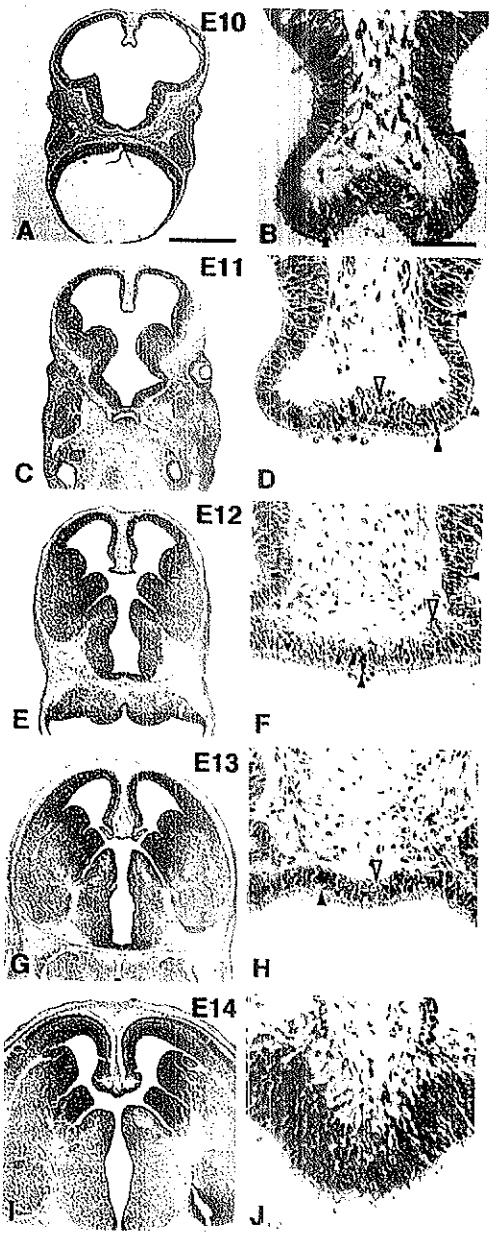


FIG. 5. High power micrographs demonstrating the cytology of apoptotic neurons (arrowheads) and their neurites (arrows) in various areas of the CNS and PNS of E12–13 mice. (A) Shows an early apoptotic neuron from prethalamic tissue along the third ventricle at E12. (B) Illustrates a multipolar neuron in the ventral spinal cord at E13. (C) Demonstrates a large/clear bipolar neuron from the trigeminal ganglion at E13; the neuron contains a relatively large nucleus with condensed chromatin clumps (the ultrastructural characteristics of a similar apoptotic large/clear cell is shown in Fig. 2). (D) Shows two labelled axons continuous with the two labelled cell bodies from which they are derived; the cells, which are located in a cervical dorsal root ganglion (E13), contain both pyknotic nuclei. Note the AnxV-biotin negative embryonic erythroblasts (open arrowhead). Micrographs A, B, and D are taken from paraffin sections, while C is taken from a semithin section. Scale bars in A–C and E indicate 19 μm, 25 μm, 18 μm and 28 μm, respectively.

can be detected by biotinylated annexin V, a relatively small proportion of the total of apoptotic neurons belongs to the category of early apoptotic neurons.

Phagocytosis of apoptotic neurons

The quantitative data presented above demonstrate that biotinylated annexin V does not bind to all apoptotic neurons, but the results do not show whether this partial lack of labelling is due to a limited binding capacity of annexin V and/or to a covering of the PSs at the outside of the apoptotic neuron. The most conceivable cause of a covering of the PSs on the outer leaflet of the plasma membrane is phagocytosis. Therefore, we investigated this process in the trigeminal ganglia of E12 and E13 mice following intracardial injection of AnxV-biotin. Ultrastructural analysis revealed that unlabelled apoptotic neurons were frequently located inside phagosomes, whereas annexin V labelled neurons were not. Even labelled late apoptotic cell debris with a disrupted plasma membrane and swollen mitochondria was not observed inside phagocytes. Instead, the annexin V stained apoptotic neurons were frequently surrounded by viable cells that were selectively labelled at the part of their plasma membrane that was directly adjacent to the dying cells (Fig. 8A). In this configuration, direct contact between the labelled membranes of the dying neurons and the surrounding cells was sporadic. In contrast, analysis of E12 progression zone and E13 degenerating interdigital tissue of mouse embryo limbs, which were studied for comparison, showed that annexin V does not hinder phagocytosis *in vivo per se*. Namely, labelled apoptotic cells were located in phagosomes in the limb mesoderm and the direct contact between the labelled membranes of the dying and the surrounding cells was much more extensive (Fig. 8B). These differences suggest that the partial lack of annexin V labelling of apoptotic neurons is due to a covering of their PSs by phagocytes, and that different mechanisms may be utilized in neuronal tissue and limb mesoderm to remove apoptotic cells from the environment.

Discussion

Classical experiments on avian embryos such as grafting (Hollyday & Hamburger, 1976) and removal (Hamburger, 1934) of chick embryo limb tissue, which can hardly be achieved in mammalian systems, have yielded invaluable information about the matching of the number of neurons to the peripheral target size and about the role of neurotrophic factors in neuronal development. Although valuable knowledge about neuronal cell death and development will probably continue to come from research on chick embryos, recently scientific input regarding neuronal development has started to derive from studies in mutant mice (Albers *et al.*, 1994; Klein, 1994; Merry *et al.*, 1994; Lo *et al.*, 1995; Furuta *et al.*, 1997). These studies have focused mainly on cell death from E15 to P10. Therefore, in the present study, we utilized the sensitive marker biotinylated annexin V and we investigated the occurrence of programmed cell death during early development of the mouse nervous system (E9–14).

FIG. 6. Time series of transverse sections through the telencephalon from E10 to E14. Note the prominent apoptosis in the velum transversum in the higher magnifications in the right column (B, D, F and H: arrowheads) with peak levels at E11 and E12. In addition, note the phagocytes containing unstained pyknotic cells and debris (D, F and H: open arrowheads). Scale bars in A and B indicate 1.1 mm and 0.11 mm, respectively.

Annexin V binds to apoptotic neurons: technical considerations

In the present study, we demonstrated unequivocally that annexin V linked to a marker such as biotin or OG can label apoptotic neurons and their extensions. The possibilities and limitations of this technique were investigated both *in vitro* and *in vivo*, both at the light microscopic and electron microscopic level, and both qualitatively and quantitatively. In the *in vitro* experiments, apoptosis was induced by NGF withdrawal, which is a well established method to evoke neuronal apoptosis (Burde *et al.*, 1980; Barde, 1989; Rimon *et al.*, 1997). We showed that annexin V binds both somata and extensions of cultured DRG neurons starting 6 h after NGF deprivation, and reaches high levels after 10–15 h of growth factor withdrawal. The fact that the nuclear chromatin of the labelled neurons was heavily labelled by Hoechst 33342 while their plasma membrane was intact, confirmed that the annexin V labelled neurons were indeed apoptotic. Electron microscopic analysis, which still stands as one of the most important methods for the identification of apoptosis, demonstrated that annexin V can also bind to both the cell body and the dendritic and axonal extensions of apoptotic neurons *in vivo*; many annexin V-stained neurons in the DRGs and trigeminal ganglia showed the typical morphological characteristics of apoptosis including chromatin condensation and cell fragmentation. It is worthwhile to notice that the presently explored annexin V labelling technique is the first to allow the identification of apoptotic neuronal extensions, and that, in this respect, it stands out against known nuclear apoptosis markers such as the TUNEL method (Gavrieli *et al.*, 1992). In addition, it was demonstrated that the method is sufficiently sensitive to detect early neuronal apoptosis both with respect to the cellular process itself and to the course of CNS and PNS development. In fact, with the use of nuclear neuronal staining methods, such as Bisbenzimide, internucleosomal degradation of DNA, a biochemical hallmark of apoptosis (Wyllie, 1980), does not start earlier than 18 h after NGF deprivation in the *in vitro* assay (Deckwerth & Johnson, 1993), indicating that annexin V is much more sensitive for detecting early neuronal apoptosis. Moreover, using annexin V in the *in vivo* preparation, we were able to detect apoptotic neurons in lumbal DRGs as early as E11, whereas with the use of the TUNEL method neuronal apoptosis can be detected not earlier than E12 (White *et al.*, 1998).

None the less, several limitations of the method have to be addressed. First, the method is not specific for apoptosis under all circumstances. Some of the cultured DRG neurons that were positively labelled with annexin V were also positively stained with the use of PI. As the plasma membrane of both the perikaryon and neurites of these annexin V labelled cells were not intact, it seems likely that these neurons were not apoptotic, but necrotic. Albeit probably of the postapoptotic- or secondary necrotic type, as the nuclei of these cells were showing the characteristics apoptosis, i.e. pyknosis and fragmentation (Darzynkiewicz *et al.*, 1997). Similarly, annexin V has also been demonstrated to label necrotic, non-neuronal tissues (Koopman *et al.*, 1994). Thus, annexin V markers may be used in brain preparations as in the present *in vivo* experiments in which necrosis hardly occurs, but control experiments such as electron microscopic analysis may still be needed for confirmation. Another restriction of the annexin V detection method of apoptosis pertains to its sensitivity. By comparing the number of pyknotic neurons that were and were not labelled for annexin V in the DRGs Th6–8 and the trigeminal ganglia from E11 to E13, we were able to demonstrate that annexin V markers cannot label more than half of the cell bodies of all late apoptotic neurons. Because our electron microscopic

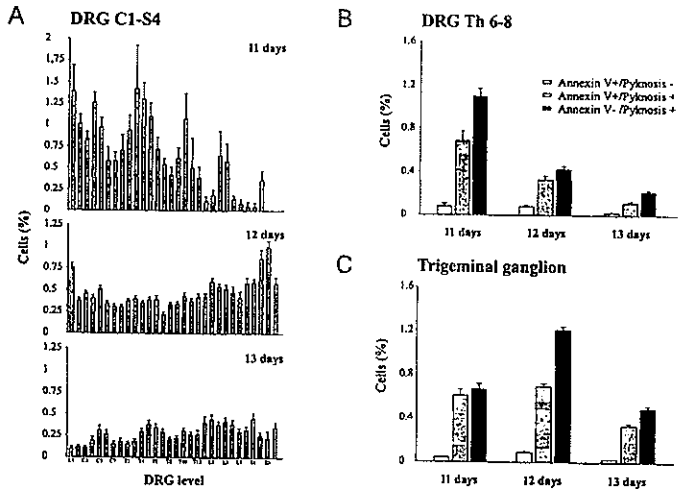


FIG. 7. Quantification of apoptotic neurons in the DRGs (A and B) and trigeminal ganglion (C) at E11, E12 and E13. (A) Demonstrates a spatiotemporal shift in the occurrence of apoptosis in the DRG; at E11 the most prominent labelling occurs in the cervical and thoracal dorsal root ganglia, whereas at E12 and E13 the lumbal and sacral levels contain more apoptotic neurons. The numbers in A indicate averages and standard errors of the mean of apoptotic DRG neurons as detected by AnxV-biotin labelling. (B,C) Show the percentages of apoptotic neurons for DRG Th6-8 and the trigeminal ganglion for E11, E12 and E13; for this quantification the dying neurons have been differentiated into those that are labelled with annexin V but not pyknotic (i.e. early apoptotic), those that are labelled and pyknotic (i.e. late apoptotic), and those that are pyknotic but not labelled (i.e. late apoptotic and presumably phagocytosed). Note that in general about half of the apoptotic neurons are not labelled with annexin V; as deduced from our electron microscopic analysis (see Fig. 8) this absence of labelling presumably results from the fact that these apoptotic cell (remnants) are located inside phagosomes.

analysis indicated that unlabelled neurons with a pyknotic nucleus were usually located inside phagosomes, it appears likely that this partial lack of labelling is due to a covering of the PSs on the outer leaflet of the apoptotic neuron. Apparently, the annexin V molecules with a weight of 35 kDa are not able to penetrate through the membranes of the phagocytes of neurons. Similarly, we have been unable to detect dying Purkinje cells in the third postnatal week of *lurcher* mice following intracardial injection of biotinylated annexin V (De Zeeuw *et al.* unpublished observations), even though their Purkinje cells die during this period due to apoptosis (Norman *et al.*, 1995). As we do get labelling of apoptotic Purkinje cells in the *lurcher* when we inject the annexin V marker directly into the brain, we assume that the annexin V molecules cannot pass through the blood–brain barrier. Thus, intracardial perfusion of annexin V markers can be conveniently used for the detection of apoptotic neurons that are not engulfed by phagocytes during prenatal and early postnatal development, but not for the detection of neuronal apoptosis after the process of phagocytosis has begun or after the blood–brain barrier formation has been completed (i.e. for mice P7–14; Brett *et al.*, 1995).

Neuronal apoptosis during early development: I. CNS

In the CNS cell death generally peaked at E12 during early development. During this stage, programmed cell death in the CNS was characterized by the occurrence of clusters of apoptotic neurons and a relative lack or absence of apoptotic axons. The occurrence of the clusters was particularly prominent in brain areas that undergo a dramatic morphogenetic change. For example, in the cerebellum, the Purkinje cells start to migrate from the rhombencephalic walls along the fourth ventricle to the area that will become the cerebellar cortex (present study; Altman & Bayer, 1985), and in the connection area between the eye and the brain, the optic nerve is trailing the degenerating optic stalk (present study; Silver & Hughes, 1973). Thus, it appears that in the CNS the clustered distribution of apoptosis plays especially an important part in areas of morphogenesis. Recently, evidence is gathering that the process of morphogenesis of the CNS is being controlled by the transient expression of particular genes. Furuta *et al.* (1997) demonstrated for example that cell death in the velum transversum is most likely under control of simultaneously expressed members of the BMP gene family and *Msx-1*.

Apart from clustered cell death, we also frequently observed dispersed cell death in the CNS. This form of apoptosis was especially prominent in the rapidly expanding rostral parts of the telencephalic hemispheres, raising the possibility that this distribution pattern of cell death is spatiotemporally linked with neuronal proliferation. Blaschke *et al.* (1996; 1998) also associated apoptosis in the developing cortex with neuronal proliferation, and suggested that these apoptotic events are linked with phenotype selection of clonally expanding neurons and the initiation of postmitotic neuron generation. An alternative or additional explanation for dispersed cell death in the developing neocortex may be that it serves to delete cells carrying mutations, which are likely to be generated in highly proliferative regions, and/or that it helps to regulate cell numbers (Thomaidou *et al.*, 1997). The latter hypothesis is supported by a recent study of Keane and coworkers (1997), which showed that targeted deletion of caspase-3 in mice results in a deficiency in apoptosis of proliferating cells and hypertrophy of the brain.

In sum, based upon the present data, we conclude that it appears a likely working hypothesis that clustered and dispersed cell death in the CNS may be particularly associated with morphogenesis and neuronal proliferation, respectively.

Neuronal apoptosis during early development: II. PNS

Whereas in the CNS apoptosis during early development may be most relevant for the control of morphogenesis and neuronal proliferation, the dispersed cell death we have documented in the PNS may play a crucial part in quantitative matching of phenotypically distinct cells with their afferent and efferent projection areas (Buchman & Davies, 1993; Coggeshall *et al.*, 1994). This hypothesis predicts that during specific postmitotic time windows neuronal cell types with distinct morphologies and functionalities compete for limited amounts of neurotrophic factors to survive (Davies, 1994; White *et al.*, 1996). The presently observed spatiotemporal distribution pattern of apoptosis during early development of the PNS is compatible with this hypothesis. Our results obtained from both ultrathin and

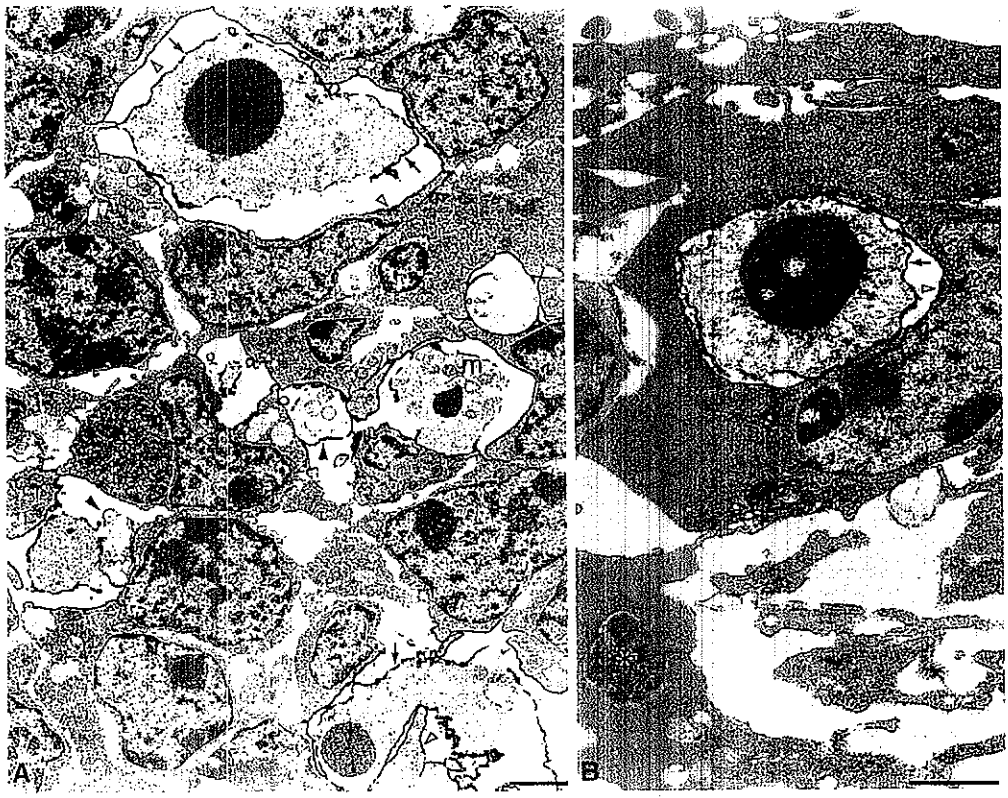


FIG. 8. Phagocytosis of neuronal and non-neuronal tissues. (A) Illustrates AnxV-biotin-labelled apoptotic trigeminal ganglion neurons (arrows) with largely intact and often clustered mitochondria (m), diminished electron density of their cytoplasm, many free ribosomes, a dilated endoplasmatic reticulum, and blebbing of their plasma membrane. Note that unlabelled neuronal debris (asterix), but not AnxV-biotin-labelled neuronal components, occur inside phagolysosomes. All annexin V labelled neurons are surrounded by an annexin V labelled plasma membrane of a viable cell (open arrowheads). Black arrowheads indicate stained apoptotic bodies with a disrupted plasma membrane and swollen mitochondria suggesting the presence of secondary necrosis. (B) Illustrates the process of phagocytosis in the progression zone in E12 limb tissue. In this mesodermal tissue both unstained debris (asterix) and AnxV-biotin-labelled cell fragments (black arrow) are located in phagocytes. As observed in neuronal tissue, the labelled plasma membrane of apoptotic cells is often surrounded by AnxV-biotin positive membranes of viable cells (open arrowhead). However, it should be noted that the total of gaps in between the membranes of the apoptotic cell and the phagocyte is much smaller than in neuronal tissue. Scale bars in A and B indicate 1.9 μm and 2.9 μm, respectively.

semithin sections indicated that all apoptotic neurons during early development of the PNS are part of the large clear group of neurons, and our quantitative light microscopic analysis indicated that the rostral (C1–Th8) and caudal (Th9–S4) DRGs show their peak level of apoptosis at E11 and E12–13, respectively. As Lawson & Biscoe, 1979) demonstrated that these neurons at these rostrocaudal levels have just reached their postmitotic phase during these periods, it appears more likely that they become apoptotic due to neurotrophic-based selection than to processes that accompanies morphogenesis, which often involves proliferating cells. This notion is further supported by the fact that small dark cells, which are also present in the peripheral ganglia during this period, but in a proliferative phase (Lawson & Biscoe, 1979), were never labelled with annexin V and did not show any sign of apoptosis.

The mechanism by which this early quantitative matching may come about presumably involves several growth factors. *In vitro* experiments have shown that neurons of the large clear group are responsive to brain-derived neurotrophic factor (BDNF) and neurotrophic factor 3 (NT-3) (Davies *et al.*, 1986). As NT-3 and/or BDNF are expressed in the projection areas of the trigeminal ganglion and DRGs during the appropriate time periods (Maisonpierre *et al.*, 1990; Davies *et al.*, 1991; Coggeshall *et al.*, 1994), apoptosis in the peripheral ganglia may indeed be mediated by these trophic factors. NGF on the other hand probably does not play a prominent part in the control of apoptosis during early development, because trigeminal ganglion neurons are not responsive to NGF until E14 (Buchman & Davies, 1993), which is 2 days after they show their peak of apoptosis (present study). Moreover, neurons of the large clear group *in vitro* are not responsive to NGF either (Davies *et al.*, 1986).

Thus, the spatiotemporal distribution of apoptosis during early

development of the PNS as observed in the present study is fully compatible with an early selection of neurons of the large clear phenotype, and possibly linked with the competition of these cells for limited amounts of NT-3 and/or BDNF in their projection areas.

Phagocytotic clearance of apoptotic neurons

The present ultrastructural data indicated that annexin V labelled neurons were never located inside phagosomes. This observation raises the questions as to whether neurons are being processed within phagosomes too quickly to be detected and/or whether annexin V blocks the process of phagocytosis. We assume that the process of neuronal intraphagolysosomal degradation is sufficiently slow to be detected following intracardial injection of biotinylated annexin V, because numerous non-labelled neuronal components were observed inside phagosomes in all our electron microscopic studies (see also Innocenti *et al.*, 1983; Ferrer *et al.*, 1990). Considering the amounts of neurons that can be removed within a single day, as observed in our quantifications of apoptotic neurons in the DRGs and trigeminal ganglia, it can be expected that the phagocytotic removal time of apoptotic neurons does not differ substantially from that of non-neuronal tissues (cf. van den Eijnde *et al.*, 1997a). Both *in vitro* and *in vivo* studies of non-neuronal tissues suggest that the time necessary for phagocytotic ingestion varies from 15 to 30 min (Falasca *et al.*, 1996; van den Eijnde *et al.*, 1997a,b). The second explanation, i.e. the possibility that the binding of annexin V to PSs itself inhibits the phagocytosis of neurons, appears more likely. Such an inhibition has already been shown for non-neuronal tissues. Fadok and colleagues (1992b) originally demonstrated that exposure of PS on the surface of apoptotic lymphocytes can trigger specific recognition and removal by macrophages, and more recent *in vitro* studies demonstrated that annexin V can inhibit phagocytosis of muscle cells and blood cells by interfering with the PS dependent death signalling mechanism (Bennet *et al.*, 1995; Bratosin *et al.*, 1997). As we sometimes observed uningested labelled material with a postapoptotic–necrotic appearance in the peripheral ganglia of animals that were intracardially perfused with annexin V, but not in our controls that were perused with HEPES buffer (data not shown), it appears indeed possible that annexin V interferes with the process of neuronal phagocytosis. In fact, it may even be possible that this interference is stronger for neurons than for other tissues, because we frequently observed labelled apoptotic cellular components inside phagosomes in the limb buds of mouse embryos. This difference may indicate that phagocytosis of at least some non-neuronal tissues, in casu limb mesoderm, is not as dependent on the exposure of PSs as that of neuronal tissue. For example, it may be possible, as demonstrated by Fadok and colleagues (1992a) for macrophages of blood cells, that some non-neuronal cell types use vitronectin receptors instead of or in addition to PS receptors for the initiation of phagocytosis. Another observation also supports the possibility that the mechanisms of phagocytosis in the nervous system differ from those in other tissues. We observed in both the peripheral ganglia and the limbs that the plasma membranes of viable cells (i.e. potential phagocytes) were annexin V positive at the surface apposed to the labelled apoptotic cells. However, whereas the labelled plasma membranes of the viable cells were always directly adjacent to most of the membrane of the apoptotic cells in the limbs, in the nervous system both plasma membranes were consistently separated by a wide extracellular gap almost surrounding the entire apoptotic neuron. As the pattern of annexin V labelling in both adjacent membranes points towards a possible plasma membrane exchange, which can be one of the initial events in the process of phagocytosis (Foa *et al.*, 1985), it may indeed be possible that the phagocytotic removal of neurons differs from that of other cell types.

Conclusions

In short, in the present study we show by probing for PS exposure with annexin V that during early mouse embryogenesis apoptotic neurons expose PS at their outer plasma membrane leaflet, irrespective of the spatiotemporal localization or the differentiation status of the cell. Apoptotic neurons were observed to be organized in clusters in the central nervous system or apparently randomly present, mainly in the DRGs and trigeminal ganglion. These modes of cell death may be linked with morphogenetic events in the CNS, and in the PNS with, presumably NT-3 and BDNF-mediated, matching of numbers of postmitotic neurons of the large clear group with their projection areas. The presence of annexin V positive cells in phagocytes in limb tissue and the absence of such labelled ingested cells in neuronal tissue suggest a possible important function for PSs in the phagocytotic clearance of apoptotic neurons during development.

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Abbreviations

Annexin V-biotin, annexin V conjugated to biotin; Annexin V-OG, annexin V conjugated to Oregon Green; BDNF, brain-derived neurotrophic factor; C, cervical; CNS, central nervous system; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DRG, dorsal root ganglion; E, embryonic day of development; EM, electron microscopical; L, lumbal; NGF, nerve growth factor; NT3, neurotrophic factor 3; P, postnatal day of development; PNS, peripheral nerve system; PS, phosphatidylserine; S, sacral; T(h), thoracal.

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Immunosuppressive effect of annexin V on LPS or anti-CD40 activated murine B lymphocytes

Stefan Maarten van den Eijnde#§12, Adri van Oudenaren*1, Katarina Radoševic*, Christl Vermeij-Keers§¶, Francoise Russo-Marie‡, Waander L. van Heerde@, Huub F. J. Savelkoul* and Chris P. M. Reutelingsperger@

MGC Dept. of Clinical Genetics, Erasmus University Medical School, Rotterdam, The Netherlands
$ Inst. of Plastic Surgery, Erasmus University Medical School, Rotterdam, The Netherlands
* Dept. of Immunology, Erasmus University Medical School, Rotterdam, The Netherlands
¶ Dept. of Anatomy, Erasmus University Medical School, Rotterdam, The Netherlands
@ Dept. of Biochemistry, Cardiovascular Research Institute Maastricht, University Maastricht, The Netherlands
‡ Hôpital Cochin, INSERM U332, Paris, France.
1 These authors have contributed equally to this study
2 Present address: Dept. of Molecular Cell Biology and Genetics, Cardiovascular Research Institute Maastricht, University Maastricht, The Netherlands

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Key words: annexin V / lymphocyte / immune response / cell death / anti-inflammatory
Corresponding author:
Huub F. J. Savelkoul
Dept. of Immunology
Erasmus University Medical School
Postbus 1738
3000 DR Rotterdam
fax: 010-4089456
tel: 010-4088142
e-mail: savelkoul@immu.fgg.eur.nl

Abbreviations: annexin V-OG: annexin V conjugated to Oregon Green **PKC:** Protein Kinase C **PLA_2:** Phospholipase A_2 **PS:** Phosphatidylserine
Footnotes:
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Summary

Annexin V is a protein mainly known from its Ca^{2+} and phospholipid binding capacity, its anticoagulant effect, and its PKC and PLA_2 inhibitory activity. In this study we have investigated the immunomodulatory action of annexin V on LPS or anti-CD40 activated murine B cells by measuring isotype production, cell proliferation and cell death. The structure function relationship of annexin V-immunomodulation was assessed by comparing wild type protein with mutant proteins with a decreased or absent ability to bind phosphatidylserine. Our data indicate that annexin V inhibits IgG_1, IgE and IgM production by LPS and anti-CD40 stimulated B cells in a dose dependent manner. This inhibitory effect is possibly due to limiting the numbers of isotype producing cells since in the annexin V treated samples cell proliferation was inhibited and the percentage of dying cells was increased. The annexin V mutant proteins showed an immunosuppressive activity that was equal to the wild type protein. In conclusion, the isotype inhibitory effect of annexin V is based on a novel phosphatidylserine binding independent mechanism, mediating cell proliferation and cell death.

1 Introduction

Of all annexins identified so far [1], annexin V is the member with the highest affinity for phosphatidylserine (PS) [2-4]. The physiologic function of annexin V is especially sought in inhibition of coagulation and inflammation [5, 6]. During pregnancy these properties may coexist. Namely, high levels of anti annexin V antibodies in patients with the autoimmune disease systemic lupus erythematosis (SLE) [7], and reduction of annexin V on placental villi [8] have been reported to be associated with thrombotic events in the placenta and loss of the allograft conceptus. Furthermore, a putative immunomodulatory role of annexin V is indicated by the high levels of antibodies against this protein that are present in patients with rheumatoid arthritis [9]. Other support for an immunomodulatory role of annexin V *in vivo* comes from studies which have shown that in addition to placental villi [10], this protein also localizes in immune privileged tissues like the brain [11], spinal cord [12] and eye [13]. We hypothesize that a possible link between (1) auto-antibodies against annexin V under pathological conditions, and (2) the expression of annexin V in certain tissues under physiological conditions may be found in an inhibitory effect of the protein on the B cell immune response.

Various investigations have shown that B cell activation plays an important role in allograft rejection [14], autoimmune diseases [15-19], degenerative events in normally immune privileged tissues [20, 21], and in infertility [22]. Purpose of this study was to investigate the effects of annexin V on immunoglobulin production by activated murine B cells. To this end, B cells were stimulated polyclonally by LPS or by anti-CD40, and the effect of annexin V on isotype production was measured in relation to cell proliferation and cell death. To study the structure-function relationship of annexin V, we tested wild type PS binding-annexin V and mutant proteins with a reduced or absent PS binding capacity. Our data indicate that annexin V inhibits isotype production by polyclonally stimulated murine B cells through limiting the numbers of isotype producing cells. This decrease in activated B cell population is most likely effected by inhibiting cell proliferation and by inducing cell death, through a PS independent interaction with the lymphocyte .

2 Materials and methods

2.1 Mice

Female BALB/c athymic nude (nu/nu) mice were purchased from Harlan Netherlands B.V., Horst.,

and were maintained at the Department of Immunology. All mice were at an age of 8-12 weeks at the start of the experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University Rotterdam.

2.2 Cell preparation

Preparation of spleen cell suspensions was described previously [23]. Nucleated cells were separated by a Histopaque-1119 gradient (Sigma, St. Louis, MO). Erythrocytes were further depleted by NH_4Cl lysis (Gey's solution). Total number of nucleated cells were counted with a Coulter Counter, Model ZM. Routinely, the percentages of contaminating T cells appeared to be less than 2 percent when employing CD3 staining in combination with flow cytometric analysis. Heparinized blood was collected from healthy donors; the samples were Histopaque treated to purify the leukocytes. NB4 and U937 cell lines were maintained on RPMI-1640 medium containing 20% Fetal Bovine Serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml).

2.3 B cell activation

B cells were activated with 50 µg/ml E. coli lipopolysaccharide (LPS, O26:B6, Difco Detroit, Mich.) or 10 µg/ml anti-CD40 (FGK-45.5, kindly provided by Dr. D. Gray). Cells were cultured in 0.2 ml DMEM media supplemented with glutamine (4 mM), sodium pyruvate (0.1 M), penicillin (100 U/ml), streptomycin (100 µg/ml), 2-mercaptoethanol (5×10^{-5} M), HEPES (25 mM) and FBS with low endogenous mitogenic activity (20%). Cells were cultured for 6 or 7 days in a 37°C, 5% CO_2 humidified incubator in 96-well flat bottom tissue culture plates (NUNCLON 167008, NUNC Brand Products, Denmark) at a concentration of 2.5×10^5/ml.

2.4 Reagents

Wild type and mutant human Annexin V were obtained by using a prokaryote expression system, as described elsewhere [24, 25]. Annexin Binding Buffer (ABB) consisted of Phosphate Buffered Saline (PBS) supplemented with 25 mM HEPES and 2% heat inactivated FBS.

2.5 Isotype-specific ELISA

Total supernatant IgM, IgG_1 and IgE levels were measured by isotype-specific ELISA as described previously [26, 27]. GAM-IgM and -IgG_1 (Southern Biotechnology, Birmingham, AL) were used at 1 µg/ml as coat and biotinylated GAM-IgM and -IgG_1 both at 0.5 g/ml as second step. RaAM-IgE (EM95) and biotinylated RaAM-IgE (Pharmingen, San Diego, CA) were used in 2 µg/ml and 1 µg/ml, respectively. Detection limits for the IgM, IgG_1 and IgE ELISA were 0.2 ng/ml, 0.2 ng/ml and 0.5 ng/ml, respectively.

2.6 Flow cytometric analysis

Cultured cells were washed twice in Ca^{2+} free buffer (PBS + 2% FBS) to wash out the excess annexin V and resuspended in ABB at a concentration of 1×10^6/ml. To measure cell death, to 100 µl of cells 10 µl annexin V-Oregon Green (annexin V-OG; 1 µg/ml; Apoptest™-Oregon Green, NeXins Research, Hoeven, The Netherlands) was added and/or 10 µl Propidium Iodide (PI; 5 µg/ml). Cells were incubated for 15 minutes at room temperature and 100 µl ABB was added just before analyzing by flow cytometry on a Becton Dickinson FACScan interfaced to a Macintosh computer running the CellQuest software (Becton Dickinson, Sunnyvale, CA). Data was collected on 5000 cells.

2.7 Confocal laser scanning microscopy

B cells were cultured in the presence of annexin V-OG (1 µg/ml) and PI (5 µg/ml) for 24 hours with or without LPS. After this culture period the cells were washed, fixed, and placed in between a slide and coverslip. The coverslip was sealed with wax, and stored at -20°C. The samples were examined using the MRC600 confocal scanning laser microscope (Biorad, Hemel Hempstead, United Kingdom), equipped with an air-cooled

Argon-Krypton mixed-gas laser and mounted onto an Axiophot microscope (Zeiss, Oberkochen, Germany). The laser scan microscope was used in the dual-parameter setup, according to manufacturer's specification, using dual wavelength excitation at 488 nm and 568 nm. Emission spectra were separated by the standard set of dichroic mirrors and barriers. Stacks of confocal planes at 0.3 μm intervals in z-distance were recorded in Kalman filtering mode, using a 40X objective.

2.8 Statistical analysis

Statistical significance was tested for with the Student's T-test (Microsoft Excel, Microsoft Corporation). P-values below 0.05 were considered as significant.

3 Results

3.1 Annexin V inhibits dose dependently isotype production by activated B cells

The effect of addition of annexin V on isotype production by polyclonally activated B cells was determined in cultures of freshly isolated BALB/c nude whole spleen cells (approximately 80% B cells and less than 2% T cells). Experiments with purified B cells from BALB/c spleen cells obtained by complement depletion using antibodies against GR-1 (granulocytes), and CD3, CD4 and CD8 (data not shown) were consistent with the experiments performed with whole spleen cell suspensions from nude mice. The isotype production by activated B cells was measured by an ELISA, after seven days of culturing. In cultures without polyclonal stimulation, isotype production was barely above detection level, irrespective whether the cells were untreated, treated with a dose of 50 μg/ml of heat-inactivated annexin V with a destroyed tertiary structure [28], or with the same dose of active annexin V (Figure 1). When cells were stimulated with LPS or anti-CD40, high levels of IgM were measured in cultures without

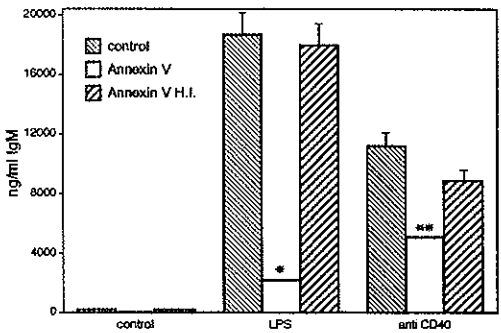

Figure 1. 5 x 10^4 LPS or anti-CD40 activated BALB/c nude spleen cells were incubated with 50 μg/ml annexin V or heat inactivated annexin V. Medium was used as control. IgM isotype production was measured in triplo in the supernatant after 7 days of culturing. In both LPS ($p = 0.0001$, asterix) and anti-CD40 ($p = 0.003$, double asterix) stimulation, significance was tested by Student T test for annexin V and heat inactivated annexin V.

annexin V, and in cultures treated with heat-inactivated annexin V (Figure 1). However, addition of wild type annexin V significantly inhibited isotype production, both by LPS ($p = 0.0001$) and anti-CD40 ($p = 0.003$) stimulated cells (Figure 1).

Further investigation showed that annexin V inhibited isotype production in a dose-dependent manner (Figure 2). A decrease in isotype production by annexin V was clearly present at a concentration of annexin V in the range of 10-25 μg/ml, whereas first inhibitory effects could be observed even at lower doses. In contrast, isotype production was not inhibited when annexin V was added to the cultures one day after polyclonal stimulation, even at high doses (Figure 3).

Combined, these data indicate that annexin V needs its tertiary structure to inhibit isotype production by B cells. Annexin V exerts its effect both on whole spleen B cells and purified B cells dose dependently by interacting with the polyclonally stimulated cells on the first day of culturing. Moreover, annexin V does not differentially affect the switching capacity of the

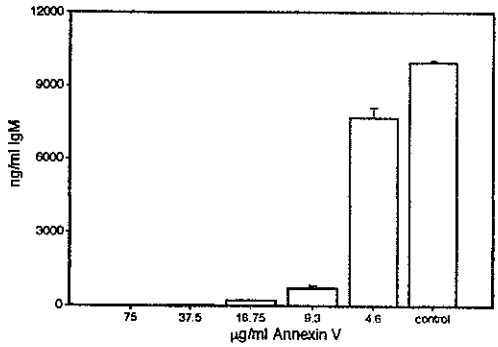

Figure 2. 5 x 10^4 LPS activated BALB/c nude spleen cells were incubated with various doses of annexin V. Medium was used as control. IgM isotype production was measured in the supernatant after 7 days of culturing. Each point is the mean ± SE of triplicate cultures.

activated B cells (data not shown). Generally, an evident effect of annexin V was observed with a dose of 10 µg/ml; IgG_1, IgE and IgM productionwas similarly reduced for both stimulatory conditions.

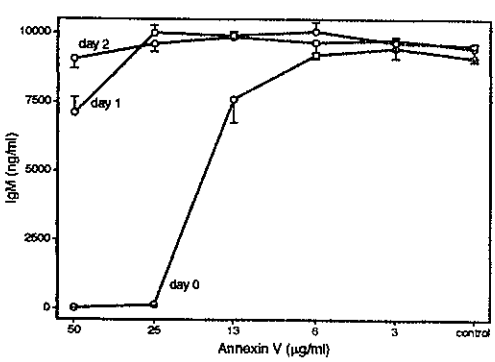

Figure 3. On day 0,1 and 2 after LPS activation, 5 x 10^4 BALB/c nude spleen cells were incubated with various doses of annexin V. Medium was used as control. IgM isotype production was measured in the supernatant after 7 days of culturing. Each point is the mean ± SE of triplicate cultures.

3.2 Modulation of B lymphocyte proliferation and cell death by annexin V

To permit the analysis of selective effects of annexin V on B cells, the possible contribution of T cells was omitted by making use of BALB/c nu/nu spleen cells. B cell response upon antigen stimulation typically involves a profound increase in cell numbers [29]. Down regulation of B cell proliferation is therefore a possible mechanism to explain inhibition of isotype production by activated B cells. To get insight in the effect of annexin V on B cell proliferation, ^{3}H-Thymidine incorporation by LPS or anti-CD40 stimulated B cells was measured three days after treatment with annexin V. Indeed, as shown in Figure 4, annexin V appeared to down regulate cell proliferation in a dose dependent manner. Compared to controls, cell proliferation was markedly decreased, showing a strong effect of annexin V at a dose of 25 µg/ml.

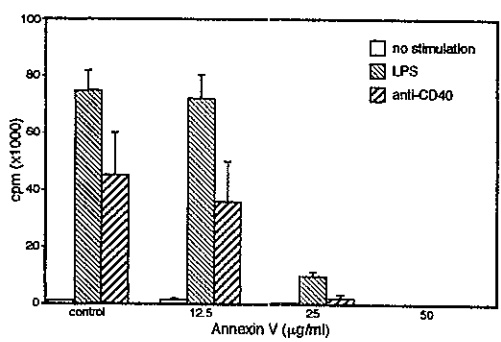

Figure 4. A dose of 50 µg/ml LPS or 10 µg/ml anti-CD40 was used to activate 5 x 10^4 whole spleen cells together with various concentrations of annexin V (50, 25, 12.5 µg/ml) and medium was used as control. After 3 days of culturing cells were pulsed for 15 hours with 1 µCi ^{3}H-Thymidine. Each point is the mean ± SE of triplicate cultures.

The observed inhibition of ^{3}H-Thymidine incorporation can be explained from a specific interference of annexin V with the mitogenic signaling. Alternatively, it may effect a decrease in—proliferating—B cell population-size by

inducing cell death. In literature, annexin V is frequently referred to as an apoptosis marker which detects the exposure of PS at the outer leaflet of dying cells [30, 25, 31]. Though, the high doses of unlabeled annexin V we have tested in the B cell-immunomodulation assay, made it impractical to use this protein also as an apoptosis marker. This is illustrated by a competition experiment where B lymphocytes—after they were biochemically forced to expose their PS molecules at their plasma membrane—were incubated with 1 μg/ml of annexin V conjugated to the fluorochrome Oregon Green subsequent to incubation with unlabeled annexin V. Whereas labeled annexin V bound 78% of the B cells in the absence of unlabeled annexin V, in similar flow cytometry experiments after addition of 3.1 μg/ml or higher concentrations of "cold" annexin V the labeled annexin V did not bind to more than 4.6 % of the B cells. Due to this limitation, in the following assays cell death numbers were determined by propidium labeling (PI), which detects—secondary or postapoptotic—necrotic cells with a compromised plasma membrane integrity [32, 33].

To determine whether annexin V induces B lymphocyte cell death, the percentages of PI labeled cells were measured 24 hours after the cells had been treated with LPS and increasing doses of annexin V. One day after antigen treatment, approximately 25 percent of the cells were PI positive and dead, irrespective the concentration of annexin V (0-25 μg/ml) (Table 1). Determining the number of dead cells two days after LPS and annexin V treatment revealed that annexin V does have the potency to trigger cell death. After a dose of 25 μg/ml or more, the cells showed a decreased forward- and sideward scatter profile, and compared to annexin V untreated controls, the numbers of PI positive cells increased, reaching levels of up to 75% PI positive cells at a dose of 25-50 μg/ml (Table 1 and Figure 5).

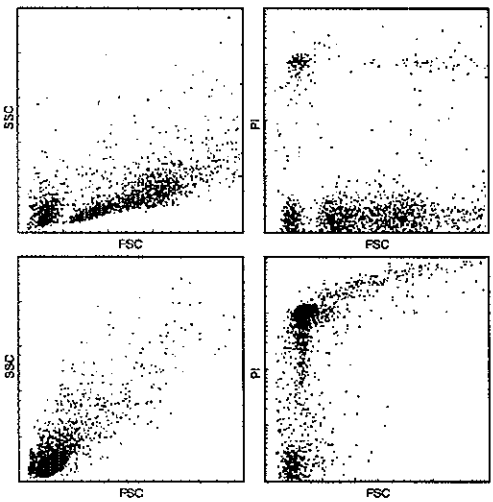

Figure 5 Flowcytometric analysis 2 days after LPS activation of BALB/c nude spleen cells, which were incubated with 25 μg/ml annexin V (lower graphs) or medium (upper graphs). Reduced scatter profile (lower left) which is already present after 12-24 hours of incubation with annexin V, is a typical feature for apoptotic cells (lower right, 71%), where in the control cells (upper right) the percentage PI-positive remains low (15%).

Apparently, B cells are more sensitive to induction of apoptosis by annexin V than other cell types. For instance, virtually all human peripheral blood cells survived the exposure for 24 hours to annexin V at doses ranging from 0-50 μg/ml. At each annexin V concentration tested, only about 2% of the cells showed a compromised integrity of the plasma membrane and stained positive for PI (Table 1).

Combined, these results support the hypothesis that annexin V may inhibit isotype production by activated B cells through limiting the numbers of isotype producing cells, effected by decreasing cell proliferation and induction of cell death. Splenic B cells appeared to be relatively susceptible to annexin V's death inducing capacity compared to human leukocytes, pointing towards a specific interaction of annexin V with B lymphocytes.

Cell type	annexin V	incubation (Hr)	dose (μg/ml)	LPS	PI+ (%)
B lymphocyte	ctrl	24	0.0	+	23
B lymphocyte	wt	24	6.3	+	25
B lymphocyte	wt	24	12.5	+	25
B lymphocyte	wt	24	25.0	+	26
B lymphocyte	wt	24	50.0	+	67
B lymphocyte	ctrl	48	0.0	+	25
B lymphocyte	wt	48	25.0	+	75
B lymphocyte	M1	24	6.3	+	24
B lymphocyte	M1	24	12.5	+	24
B lymphocyte	M1	24	25.0	+	35
B lymphocyte	M1	24	50.0	+	73
B lymphocyte	M1234	24	6.3	+	23
B lymphocyte	M1234	24	12.5	+	24
B lymphocyte	M1234	24	25.0	+	23
B lymphocyte	M1234	24	50.0	+	66
Peripheral Blood	ctrl	24	0.0	-	~2
Peripheral Blood	wt	24	6.3	-	~2
Peripheral Blood	wt	24	12.5	-	~2
Peripheral Blood	wt	24	25.0	-	~2
Peripheral Blood	wt	24	50.0	-	~2
NB4	ctrl	24	0.0	-	4
NB4	M1234	24	50.0	-	5
NB4	M1234	24	100.0	-	3
U937	ctrl	24	50.0	-	1
U937	M1234	24	50.0	-	1

The percentage of propidium iodide permeable (PI+) dead B cells was assessed via FACS analysis (5000 events) 24 or 48 hours after incubation with different doses of (mutant) annexin V in the presence or absence of LPS.

Table 1. Percentage of dead B lymphocytes, peripheral blood cells, and NB4 and U937 cells after culturing in the presence of different doses of wild type annexin V (wt), or mutant annexin V (M1 and M1234).

3.3 Uptake of annexin V

To get insight in the manner by which annexin V might effect its immunomodulatory effect on B lymphocytes, the cellular distribution of annexin V in B cells was assessed with the confocal laser scanning microscope after incubating the cells with annexin V conjugated to the fluorescent probe Oregon Green (annexin V-OG) and PI to assess plasma membrane integrity. Similar staining was observed in LPS treated and untreated (Figure 6) B lymphocytes. In the samples, cells could be categorized into four major groups based on the staining for both probes. Cells from the first group were unlabeled for both annexin-OG and PI, indicating that these cells were not apoptotic nor postapoptotic or secondary-necrotic, and neither had internalized annexin V (data not shown). The second group resembled classical apoptosis [30, 25], *i.e.*, staining for cell surface exposed PS, while the plasma membrane is intact and able to exclude PI (Figure 6A). The third group of cells showed

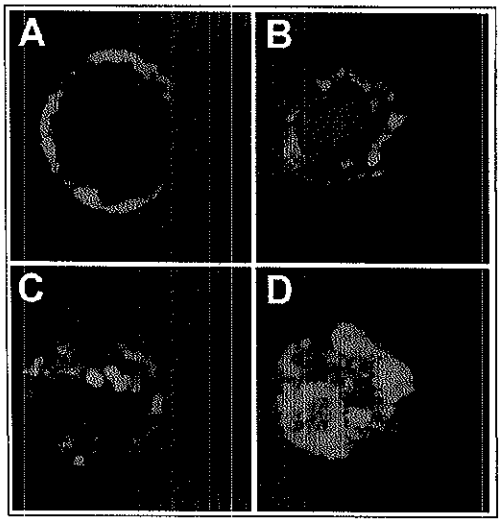

Figure 6. Representative, mid-sagittal, optical sections of B cells obtained via confocal laser scanning microscopy 24 hours after incubation with annexin V-OG at a dose of 1 µg/ml (green), and PI (red). A typical example of an apoptotic cell is shown in figure A, *i.e.* a strong staining with annexin V for PS exposed at the plasma membrane, whereas the absence of PI labeling indicates that the plasma membrane is intact. Figure B shows a cell highly permeable for PI, and strongly stained with annexin V in the cytosol and plasma membrane. Figure C and D show cells containing annexin V in the cytosol. We hypothesize that this annexin V has actively entered the cells, because the cells were having an uncompromised plasma membrane integrity, as indicated by the absence of PI labeling.

both an intense staining at the nucleus for PI, and labeling with annexin V-OG in the cytosol and at the plasma membrane. These cells may be considered as postapoptotic or secondary necrotic (Figure 6B). Cells of group four did neither show a clear labeling for annexin V-OG at the plasma membrane like apoptotic cells, nor a compromised plasma membrane integrity, as indicated by the absence of PI labeling. But these cells showed an intense staining for annexin V in their cytosol; indicating that they actively had internalized annexin V (Figure 6C and D).

3.4 The effect of annexin V on activated B cells is PS-independent

To study the structure-function relationship of annexin V's immunomodulatory effect on stimulated B cells, we determined dose response curves for two mutant annexin V-proteins with a decreased affinity for PS. One of these proteins, M1, contains a mutation in the endonexin loop of the first domain, resulting in a 50 % decrease in PS binding capacity compared to wild type annexin V protein; M1234, has mutations in all four endonexin loops, resulting in a complete loss of PS binding capacity [34].

Clearly, all three proteins were equally potent in inhibiting IgM production by the LPS activated B cells. The first effects were measurable at 10 µg/ml of protein, while hardly any B cell isotype production was left at a concentration of 25 µg/ml (Figure 7). Interestingly, the effect of M1 and M1234 on cell survival was also similar to the effect of wild type protein; one day after being

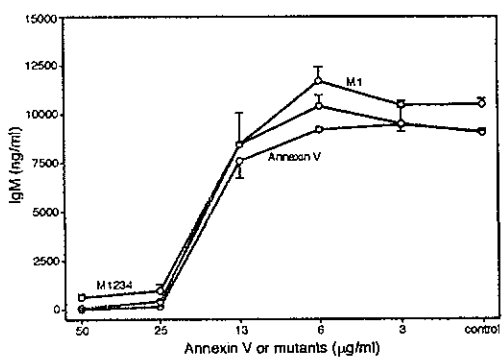

Figure 7. 5 x 10^4 LPS activated BALB/c nude spleen cells were simultaneously incubated with various doses of wild type annexin V or annexin V mutants. M1, has a mutation in the Ca^{2+} binding site in domain one, resulting in 50% decrease in PS binding capacity compared to wild type annexin V protein. The other mutant used, M1234, has mutations in its Ca^{2+} binding sites of all four domains, resulting in a complete loss of PS binding capacity. Medium was used as control. IgM isotype production was measured in the supernatant after 7 days of incubation. Each point is the mean +/- SE of triplicate cultures.

exposed to a dose of up to 25 μg/ml of mutant protein, cell survival was similar to annexin V-untreated controls, whereas at a dose of 50 μg/ml cell death levels were greatly increased (Table 1). Non B cells appeared to be inert with respect to the death inducing capacity of mutant annexin V up to concentrations of 100 μg/ml (Table 1). In summary, the overlapping immunomodulatory effects of wild type annexin V and the mutant proteins indicate that annexin V exerts its immunomodulatory effect on B cells in a PS independent manner.

4 Discussion

In this study we show that human recombinant annexin V dose dependently inhibits isotype production by LPS and anti-CD40 activated murine B cells *in vitro* by down regulating cell proliferation and by inducing cell death. This immunomodulatory activity of annexin V depends on its intact tertiairy structure and not on its phospholipid binding property, as was shown by heat-inactivated and mutant annexin V, respectively. As such this biological activity of annexin V fundamentally differs from its anticoagulant activity.

4.1 Annexin V inhibits isotype production by B cells dose dependently

In contrast to heat-inactivated annexin V, *i.e.* annexin V with a destroyed tertiary structure [28], active annexin V dose-dependently inhibited IgG_1, IgE and IgM production by both LPS and anti-CD40 stimulated B cells. Adding the cell-rescuing cytokine IL-4 in combination with annexin V did not influence isotype production, only the beneficial switching capacity could be confirmed by the increased production of IgG_1 and IgE [35]. When annexin V was added in combination with LPS or anti-CD40 at the start of culturing, dose-response curves showed a steep decline in isotype production from an apparently inactive dose to the first higher dose of annexin V. Typical thresholds for annexin V's immunomodulatory action were observed in the range of 10-25 μg/ml of annexin V. B cells that were incubated with annexin V after being cultured 24 hours in the presence of LPS or anti-CD40 did not show any response to the protein, and were potent isotype producers similar to B cells cultured in the absence of annexin V. Thus it seems that B cells are sensitive to annexin V modulation only in a limited time window around the event of stimulation.

4.2 Annexin V inhibits cell proliferation and induces cell death

Although the amount of isotype production per polyclonally stimulated B cell may vary, isotype production per assay is strongly related to cell numbers. Hence, the inhibition of the immunosuppressive effect of annexin V may be linked with the inhibition of cell proliferation. Measuring ^3H-incorporation by LPS and anti-CD40 activated cells showed that inhibition of cell proliferation is indeed one of the mechanisms by which annexin V may modulate isotype production. Namely, at the immunosuppressive-dose of 25 μg/ml, the proliferative index as measured by ^3H-incorporation was clearly reduced in the mitogen stimulated cultures.

Annexin V could bring about a reduction of proliferation by exhibiting a direct cytotoxic effect on the B cells or by inducing B cell apoptosis. Especially in highly proliferative tissues the induction of apoptosis may have a profound effect on the generation of new cells. We hypothesize that induction of cell death by annexin V does probably involve this organized mode of cell death. This notion derives from the observed delay in onset of loss of plasma membrane integrity as indicated by the uptake of PI, caused by annexin V up to the isotype inhibiting dose of 25 μg/ml. Annexin V was having no effect on the first day of incubation, but numbers of PI positive cells were reaching high levels on the second day. This late induction

of cell death indicates that the protein probably does not induce necrosis directly, which may be expected to occur within the first few hours of incubation with a toxic compound [36], but instead induces apoptosis. This organized mode of cell death is normally detectable approximately one day after triggering the process [36], and culminates into postapoptotic or secondary necrosis if these cells are not ingested rapidly by phagocytes, such as often is observed in *in vitro* assays [33, 37].

Other lines of evidence supporting a non cytotoxic action of annexin V on B cells are: (1) in the presence of 50-100 μg/ml annexin V some B cells did survive, proliferate and differentiate, and were able to produce immunoglobulines (AvO and SvdE; unpublished results); (2) incubating peripheral blood with different concentrations of annexin V showed that these cell were resistant to the proteins pro-apoptotic activity, after one day of incubation in the presence of annexin V concentrations ranging from 0-50 μg/ml, 98 % of these cells were able to exclude PI and were apparently still viable; and (3) NB 4 and U937 cell lines showed to be inert to mutant annexin V with respect to the induction of cell death up to doses of 100 μg/ml. In sum, these data indicate that annexin V probably mediates B cell-isotype production by reducing cell numbers. This cell reduction may be mediated by inhibition of cell proliferation and/or by the induction of cell death. The latter effect of annexin V seems cell type dependent, affecting B lymphocytes shortly after their activation.

4.3 The interaction of annexin V with B cells

Inhibition of proliferation and induction of cell death are important mechanisms to control the immune system [29, 36]. Recent studies have shown that cell death may even act as a double edged sword in regulating the immune system by both reducing the number of potentially immunoreactive cells [38-40] and by inducing the release of anti-inflammatory agents by phagocytes after the scavenging of apoptotic cells [41-43]. Although our study does not provide direct insight in how annexin V reverses the proliferative effects of LPS and anti-CD40 leading to a decrease in lymphocyte proliferation and activation of apoptotic pathways, it is tempting to speculate about how the protein could interfere with the polyclonal-induced B cell immune response.

An explanation for the immunosuppressive effect of annexin V on B cells could be the inhibition of intracellular protein kinase C (PKC), which is activated in LPS stimulated B cells and which modulates proliferation, differentiation and signal transduction [44]. Namely, annexin V is considered to be a potent inhibitor of PKC activity, and intracellular annexin V levels have been shown to be inversely related with the growth state of cells [45-47]. Moreover, Dubois and co-workers [48] have recently shown that phosphorylation of annexin II in the activation pathway of T cells is regulated by annexin V, potentially through inhibiting PKC activity. Although Dubois and co-workers [24] have shown that annexin V mediates PKC activity by binding PS in a Ca^{2+} dependent manner, it may be hypothesized that this protein can also inhibit PKC activity in a PS independent manner by its C terminal tail which shares homology with endogenous inhibitors of PKC, the 14-3-3 proteins [34]. Such mechanism would corroborate with our findings that destruction of the phospholipid binding property of Annexin V by site-directed mutagenesis does not result in a destruction of its ability to inhibit the B-cell immune response. The latter observation may also be explained by the studies using annexin V oligopeptides (residues 204-212) [49]. This peptide is not involved in phospholipid binding and yet exhibits immunomodulatory activities like the reduction of PGE_2 release from human fibroblasts and rat macrophages and inhibition of carrageenin-induced edema [50], inhibition of smooth muscle contraction and platelet aggregation

[51], and inhibition of neutrophil chemotaxis [52] and pyresis [53]. These activities all could be attributed to inhibiting PLA_2 activity, but mainly of the secretory, exctracellular, type of PLA_2. Studies have shown that also wild type protein can inhibit PLA_2 activity in a PS independent manner [54], including the cytosolic PLA_2 [55, 56]. Suppression of cytosolic PLA_2 may result in the inhibition of the release PGE_2 and arachidonic acid from the plasma membrane, and is likely to modulate cell proliferation and differentiation [54]. Another possibility is that annexin V activates directly death pathways in the cell.

If annexin V can inhibit the B cell immune response via inhibition of PKC or cytosolic PLA_2, a prerequisite for such a mechanism would be that the soluble protein relocates to the intracellular compartment. The confocal laser scanning experiment of B cells incubated for 24 hours with fluorescent annexin V, indeed indicates that annexin V may enter B cells in an active manner. This was shown by the strong intracellular staining for annexin V in cells not yet apoptotic, as revealed by the absence of membrane labeling with annexin V; neither were these cells (postapoptotic or secondary-) necrotic, as shown by the absence of staining for the membrane impermeable dye, PI. A putative mechanism for this internalization of annexin V may be found in the antigen processing capacity of B cells, since pathways have been shown to exist by which the contents of endocytosed particles leaks into the cytosol, possibly through an overload of the phagocytic compartment [57]. A prerequisite for internalization is that annexin V interacts with the B cell plasma membrane. Preliminary studies are in favor of a low affinity receptor for annexin V at the surface of B cells, since a 50-fold increase in cell density does not alter the incidence of cell death in each 0-50 μg/ml annexin V treated cultures (AvO, unpublished results).

Further insight of annexin V's immunomodulatory capacities and the mechanisms underlying it, may derive from the studies on annexin V transgenic mice that are underway. Such knowledge may help to unravel the role of annexin V in auto-immune disease and immune privileged tissues, and will help to further appreciate and extend the recent finding that coating of artificial intraocular lenses with annexin V inhibits the inflammatory response from the surrounding tissue [58].

Acknowledgments

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General discussion

In this thesis, annexin V plays a dual role. In the first studies, this phospholipid and calcium binding protein was used to get insight in the *in vivo* distribution of PS across the cytoplasmatic and outer leaflet of plasma membranes, and it was applied as a marker for apoptosis (chapters 2-5). In later studies, annexin V itself was subject of interest, and its putative role in the immune system was tested *in vitro*, with focus on the B cell immune response (chapter 6). The results of these two lines of research are discussed in this chapter in relation to unpublished data and recent literature.

I. Exposure of phosphatidylserine by apoptotic cells *in vivo*

a. Cell surface exposure of phosphatidylserine during embryonic development

Since each cell contains PS in its plasma membrane, annexin V applied to cryostat sections through mouse embryos was found to label all cells, irrespective the distribution of PS across both plasma membrane leaflets (chapter 2). Therefore, annexin V was injected into the blood stream of viable embryos, so that the protein would become distributed throughout the specimens via their own functioning circulation and it would only bind to cell surface exposed PS. To control for a nonspecific interaction of annexin V with mouse embryonic cells, embryos were also injected with heat inactivated annexin V-biotin (chapter 2). By heating annexin V-biotin for 10 minutes at 56°C, the tertiary structure of the protein is disturbed, and the ability to bind PS is lost (Reutelingsperger *et al.*, 1985). In later experiments, biotin conjugated annexin V with mutations in all the four calcium binding domains, named M1234-biotin, served as another more specific negative control protein (C. P. M. Reutelingsperger and S. M. van den Eijnde, unpublished data); the mutations in M1234-biotin result in a complete loss of affinity for PS molecules (Mira *et al.*, 1997). In both control situations, *i.e.* using heat inactivated annexin V-biotin and M1234-biotin, labeling was not observed in the embryos, suggesting that the staining found in embryos that were injected with intact annexin V-biotin is due to a specific interaction of annexin V with cell surface exposed PS.

Ex utero survival of mouse embryos for 30 minutes after injection with annexin V-biotin showed to be sufficient to label cells. Although most cells were unlabeled with the protein, a strong annexin V-biotin staining at the plasma membrane was observed throughout the embryos of individual cells that belonged to the ectoderm, mesoderm, and endoderm lineages. Generally, the spatiotemporal distribution of annexin V-biotin labeled cells mirrored the apoptosis patterns that were known from literature (Glücksmann, 1951), and also the left-right symmetry of the labeling patterns that was observed, pointed towards a specific interaction of annexin V-biotin with apoptotic cells *in vivo*. At the light microscopical level, many of the labeled cells were recognizable as apoptotic by their condensed nuclear chromatin (chapters 2-4). More proof for the specificity by which annexin V-biotin bound apoptotic cells came from the ultrastructural investigation of annexin V-biotin labeled cells. In ultrathin sections through embryonic limb mesoderm, cells labeled at their whole circumference were recognizable as apoptotic by morphological characteristics such as crystalline areas of ribosomes, a dilated endoplasmatic reticulum, chromatin condensation and nuclear pyknosis (chapters 2 and 4). Subsequent electron microscopical studies indicated that also annexin V-biotin labeled neurons of the ectoderm cell

lineage were showing the ultrastructural characteristics of apoptosis (chapter 5). Thus, these data from annexin V injected mouse embryos are strongly supportive of a tight regulation of PS distribution across both leaflets of the plasma membrane leading to PS exposure by apoptotic cells in regions of developmental cell death.

b. Cell surface exposed phosphatidylserine and phagocytosis of apoptotic cells

The link between apoptosis and PS exposure *in vivo* corroborates various *in vitro* experiments, which have shown that this phospholipid is an important mediator of phagocytosis of apoptotic cells (Fadok *et al.*, 1992a; Fadok *et al.*, 1992b; Bratosin *et al.*, 1997). In addition, shielding cell surface exposed PS by adding annexin V to culture media has recently been shown to inhibit phagocytosis of blood cells by "professional" macrophages (Bratosin *et al.*, 1997). In similar experiments, annexin V was found to inhibit phagocytosis of apoptotic smooth muscle cells and spermatogenetic cells by viable smooth muscle cells and Sertoli cells, respectively (Palmi *et al.*, 1995; Shiratsuchi *et al.*, 1997), which may be considered as "amateur" phagocytes (Savill, 1995). Hence, it was expected that also *in vivo* shielding of cell surface exposed PS by exogenous annexin V-biotin would lead to attenuation of the normally occurring *in situ* phagocytosis by professional macrophages and/or surrounding phagocytes (Ballard and Holt 1968; Godin *et al.*, 1995).

The ultrastructural studies of annexin V-biotin labeled neuronal cell death indeed was supportive for an *in vivo* function of PS exposure in phagocytic clearance of apoptotic cells. In neuronal ganglia from mice injected with annexin V-biotin, postapoptotic-necrotic material with a disrupted plasma membrane and swollen mitochondria was frequently observed outside phagocytes, whereas in control specimens such degraded material was only found in phagolysosomes. Furthermore, in these ganglia, labeled apoptotic neurons always seemed to be located in the interstitial compartment and were not observed in phagolysosomes (chapter 5). In contrast, in limb tissue, annexin V-biotin binding to the plasma membrane of apoptotic cells apparently did not interfere with the process of phagocytosis, because interdigital apoptotic mesodermal cells were frequently observed to be labeled, ingested and partially degraded after the same 30 minutes of *in vivo* incubation with annexin V-biotin (chapters 2 and 5). It has to be noted that the presence of membrane labeled cell material in phagolysosomes in limb mesoderm does not necessarily oppose the functionality of PS in the clearance of apoptotic cells *in vivo* in this tissue since *in vitro* studies have shown that depending on phagocyte lineage or activation status different receptors may be used to recognize the various apoptotic epitopes exposed at the dying cell (Fadok *et al.*, 1992a; Pradhan *et al.*, 1997). Combined, these results suggest that phagocyte recognition of apoptotic neuronal cells *in vivo* probably depends on surface exposed PS, whereas the recognition of apoptotic limb mesoderm is possibly also mediated by other surface epitopes.

Although the investigation of annexin V-labeled neuronal and limb tissue suggests that phagocytes in these tissues probably respond differently to covering PS at the surface of the apoptotic cell, electron microscopical data point towards some interaction between the membranes of apoptotic cells and the surrounding viable cells or phagocytes in both tissues (chapter 5). This is indicated by the annexin V-biotin staining at the plasma membrane of these viable cells only directly opposite the annexin V labeled plasma membranes of apoptotic neurons and limb mesodermal cells.

At present, two theories may explain this labeling of the adjacent membranes. In the first theory, this labeling is thought to reflect an exchange of PS exposing membrane of the apoptotic cells with the surrounding cell. Such an exchange of membrane has been observed in a model of cellular recognition of lymphocytes and their target cells (Foa *et al.*, 1985). A second theory is that in addition to the apoptotic cell—which exposes PS across the whole perimeter—also the surrounding cell may expose PS, specifically at that membrane surface involved in endocytosis of the apoptotic cell. This cell local surface exposure of PS may function in increasing the concentration of secretory phospholipase A_2 ($sPLA_2$) activity in this area (Hack *et al.*, 1997), which may be a prerequisite for membrane-elongation during the process of engulfment (Lennartz *et al.*, 1997). To test these theories, PS exposing cells may be locally injected into, for example, limb tissue. Subsequently, it can be determined immunohistochemically whether surface molecules of these injected cells have become located at the surface of embryonic phagocytes, which may point towards the presence of membrane exchange. Alternatively, in annexin V-biotin perfused embryos material may be injected which does not bind this marker, such as apoptotic cells covered with unlabeled annexin V or latex beads. If this material becomes ingested, and the phagocyte is locally labeled with annexin V-biotin this is strongly suggestive for local disturbance of PS plasma membrane asymmetry during phagocytic activity.

In addition to apoptotic cells, in the serially sectioned annexin V-biotin injected embryos also non-apoptotic cells belonging to two lineages were found to be labeled across the full perimeter of the plasma membrane. These cells were myoblasts and myocardioblasts, and megakaryoblasts riping into megakaryocytes. By their clearly non-apoptotic morphology, these cells probably will not interfere with the use of annexin V-biotin as a marker of apoptosis (see below). However, these data may be of interest with regard to studies of PS exposure and phagocytosis. It is suggested that these viable cells and apoptotic cells share a need for close contact of plasma membranes to fuse into myoblasts, to form intercalated disks, keep platelets together, or to become ingested by phagocytes, respectively (chapter 2). A possible functionality of PS exposure by viable cells was also observed in the gut of *Drosophila* pupae. During the P4-P8 stage (Bainbridge and Bownes, 1981) the embryonic gut degenerates through apoptosis and the adult gut is generated and forms a syncytium around these degenerating cells. At this stage of development a peculiar staining pattern is seen of degenerating embryonic gut surrounded by stained fusing adult gut cells with in between an unstained basement membrane (S. van den Eijnde, C. Reutelingsperger and E. Baehrecke, unpublished results). These examples from mammal and insect suggest that PS may function as a sort of phylogenetically conserved molecular glue for plasma membranes.

Evidently, the non-apoptotic PS exposing cells also differ from apoptotic cells in the sense that only the latter should be cleared by phagocytes. This implies that factors exist which regulate the difference in fate between viable and apoptotic PS exposing cells. An explanation is that surface molecules additional to PS mediate phagocyte adherence and ingestion of apoptotic cells. In this context these auxiliary epitopes are likely to be absent at the surface of the viable cells that expose PS (Pradhan *et al.*, 1997). Another explanation may come from the observation that in areas of developmental cell death (Ballard and Holt, 1968) as well as during pathological apoptosis (Savill, 1995), there is often an accumulation of specialized macrophages in time. This may indicate that in addition to short distance signaling, apoptotic cells probably are also capable to attract

phagocytes via signalling over longer distances. By lacking such signals, viable cells may remain unnoticed by the phagocytes during their PS exposing-phases of differentiation.

More insight in putative *in vivo* functions of PS may derive from future electron microscopical studies of annexin V-biotin labeled cells at different gestational ages and after longer incubation times with annexin V-biotin. More specifically with regard to the validation of the role of PS in phagocytosis *in vivo*, quantitative data is required, which may include the comparing of numbers of phagosomes between annexin V-biotin injected specimens and non-injected controls in for example limb mesoderm and neuronal tissue. Definitive proof for the involvement of PS in the process of phagocytosis, however, will possibly only derive from experiments in animals lacking PS receptors or having a deficiency in the ability to regulate the distribution of PS across both leaflets of the plasma membrane. Such studies are awaiting the identification and cloning of PS receptors as well as further characterization of the mechanisms governing the plasma membrane topography of PS.

c. Cell surface exposure phosphatidylserine: a hallmark of apoptosis

The annexin V-biotin distribution studies in viable mouse embryos strongly suggest that the presence of PS in the outer layer of the plasma membrane is a common feature of apoptosis (chapter 2). This corroborates with *in vitro* studies, that show the ubiquity of the phenomenon of PS exposure during apoptosis (Martin *et al.*, 1995). The insight that annexin V-binding was not restricted to cells of a particular lineage, combined with data from literature which showed that also insect hemocytes respond to PS by phagocytosis (Ratner *et al.*, 1986) lead to the hypothesis that the redistribution of this phospholipid during apoptosis could be considered as a hallmark of the phylogenetically conserved process of apoptosis. If this were correct, the regulation of PS plasma membrane asymmetry, leading to PS exposure during apoptosis would probably be shared between evolutionary distant species. We tested this hypothesis by injecting annexin V-biotin into the circulation of chick embryos, and into the hemolymph of *Drosophila* pupae. The results obtained from both species were in line with previous observations in mouse embryos (chapter 2). In avian and insect almost all labeled cells were morphologically recognizable as apoptotic based on the pyknotic nucleus, and were located in regions of developmental cell death (chapter 3; van den Eijnde *et al.*, 1998a). Subsequent *in vivo* studies have shown that also in adult mice physiological apoptosis was labeled during for example follicular maturation (van Engeland *et al.*, in press). Furthermore, using annexin V as a marker, apoptosis has been monitored in lymphosarcoma after *in situ* gamma-irradiation or chemotherapeutic treatment (S. van den Eijnde and N. de Both, in prep). In addition, with this protein pathological cell death was detected in transplanted hearts, and in the liver and lymphomas after FAS ligation and treatment with cyclophosphamide, respectively (Blankenberg *et al.*, 1998). These results, plus the finding that also plant cells expose PS during apoptosis (O'Brien *et al.*, 1997), are strongly supportive for denominating PS exposure as a hallmark of apoptosis.

The insight that apoptotic cells commonly expose PS during apoptosis is of interest with regard to studies of signaling between apoptotic cells and their surrounding; but also the combined hallmark nature of this phenomenon plus existence of a specific probe for its detection may provide a new method for the detection of apoptotic cells *in vivo*.

After more than a century, the method of reference to determine whether a cell is apoptotic, is still studying the presence of morphological hallmarks. Another classical method, which is often used

in studies by developmental biologists is the staining of regions of superficial apoptosis in whole unfixed developing insect and vertebrate specimens using vital dyes (Saunders Jr *et al.*, 1962). These dyes, such as neutral red, acridine orange and Nile blue sulfate probably labels apoptotic cells indirectly by staining phagolysosomes (Steller *et al.*, 1994; White *et al.*, 1994; Mori *et al.*, 1995). A breakthrough in apoptosis research was the development of a marker based on the detection of a biochemical alteration of cells during execution of the death program, *i.e.* internucleosomal DNA fragmentation (Wyllie, 1980). Using a terminal deoxynucleotidyl transferase, deoxy-nucleotides conjugated to marker molecules such as biotin, FITC or alkaline phosphatases can be incorporated in the DNA gaps, yielding it possible to stain apoptotic cells in paraffin, cryostat and ultrathin sections (Gavrieli *et al.*, 1992; Wijsman *et al.*, 1993; Pellier *et al.*, 1994).

In comparison to the established methods of *in situ* staining of apoptotic cells, labeling apoptotic cells by perfusing living embryos with annexin V-biotin differs in the sense that via this method apoptotic cells can be stained in whole embryos, as well as in paraffin and ultrathin sections (chapter 4; Table 1). With regard to vital dyes, apoptotic patterns that were visualized in whole embryos with Nile blue sulfate overlapped with those stained with annexin V-biotin. Technically, the main advantage of annexin V-biotin over the use of vital dyes is that annexin V-biotin stained specimens can be fixed and preserved for later study, while the vital dye staining in unfixed specimens needs to be evaluated directly. Due to their simplicity and avidity, however, vital dyes may still be preferred in some experimental studies, especially if only patterns of superficial cell death need to be evaluated. Comparing the staining for PS exposure with the detection of apoptosis associated DNA fragmentation in a double labeling experiment using the annexin V-biotin labeling method and the TUNEL assay, only partially overlapping staining was observed in the interdigital degenerating tissue (chapter 5). Three groups of labeling could be discerned: annexin V+/TUNEL-, annexin V+/TUNEL+, and annexin V- /TUNEL+. Pyknotic late apoptotic cells were found to be both annexin V+ and TUNEL+, or only TUNEL+. It is supposed that the latter were located in phagocytes during perfusion with annexin V-biotin. This is based on investigations of annexin V-biotin stained material in ultrathin, semithin and to a lesser extent also paraffin sections, which showed that unlabeled apoptotic material was normally located in phagolysosomes (chapters 2, 4 and 5). In addition, it seems that with the TUNEL method especially this ingested material is labeled, maybe because of an advanced DNA fragmentation of apoptotic cells within the phagolysosome, possibly due to lysosomal enzymes (Collins *et al.*, 1997). In contrast, early apoptotic cells were normally not observed labeled for DNA fragmentation, whereas such cells with a rounded-off appearance and first signs of chromatin condensation were labeled with annexin V-biotin (chapter 4). This suggests that *in vivo*, PS exposure precedes DNA fragmentation as detected via the TUNEL method. This temporal pattern of apoptotic events corroborates *in vitro* studies which have shown that PS exposure is a relatively early event, preceding nuclear pyknosis and DNA fragmentation (Ashman *et al.*, 1995; Martin *et al.*, 1995; Verhoven *et al.*, 1995; Castedo *et al.*, 1996; Rimon *et al.*, 1997; Stuart *et al.*, 1998).

The choice of apoptosis marker needs to be determined by the scientific question to be addressed. The main advantages of the *in vivo* labeling method with annexin V-biotin is the detection of apoptotic cells throughout the developing specimens which can be achieved via a relatively simple injection technique, and can be evaluated at the light microscopical and electron microscopical level. This feature may especially be valuable in studies where it is suspected that apoptosis is

abnormally regulated, but the spatiotemporal coordinates or cell types affected are unknown, which for example may be the case in mutant animals with a disturbed cell signaling or patterning.

Also studies of neuronal cell death, in particular with regard to the projection areas of apoptotic cells, may benefit from labeling with annexin V-biotin, since via this labeling method the whole perimeter of apoptotic cells is labeled, including the neurites (chapter 5). If, however, research pertains the study of apoptosis in archived material, perfusion with annexin V is no option, and staining for DNA fragmentation is probably then the method of choice.

	Routine Histology	Vital Dyes, e.g Nile Blue Sulfate	Detection of DNA fragmentation, e.g TUNEL method	Detection of cell surface exposure of PS with annexin V
Whole Mounts	-	+	-	+
Light Microscopy	+	-	+	+
Electron Microscopy	+	-	+	+
Archived material	+	-	+	-
Primarily detects:	Pyknotic cells	Phagolysosomes	Pyknotic cells	Early apoptotic and also pyknotic cells, before phagocytosis

Table 1: An overview of frequently used apoptosis detection methods, and some of their functional characteristics.

In most *in vivo* studies of apoptosis, obtaining qualitative data is sufficient. Though, if quantitation of apoptotic cells is required, it has to be considered that exogenous annexin V does not label cells that were already located in phagocytes during perfusion with the protein. In a study of neuronal apoptosis in early mouse embryos, it was shown that approximately half of the cells were annexin V-biotin labeled early and late apoptotic, while the other half of the apoptotic cells were unlabeled late apoptotic and presumably located in phagocytes (chapter 5). Although these proportions were rather constant in nervous ganglia between inbred embryos that were matched for developmental age and location, preliminary data from the study of other spatiotemporal locations indicate that the ratio of annexin V-biotin labeled vs. unlabeled phagocytosed cells is certainly not a constant value in all tissues (S. van den Eijnde, unpublished data). This may not come unexpected, since this ratio relates to the dynamics of phagocytic clearance and degradation of apoptotic cells, which are likely to be influenced by phagocyte lineage, number of phagocytes and load of apoptotic cells. Thus, as long as the dynamics of removal of apoptotic cells can be summarized as *being a rapid process*, it will be difficult to obtain accurate quantitative data of the occurrence of apoptosis, irrespective the marker used.

II. Putative *in vivo* functions of annexin V

a. Annexin V expression in mouse embryos

Since previous studies had shown that annexin V was endogenously present during development of both early fish embryos (Farber *et al.*, 1997) and post E13 mouse embryos (Rahman *et al.*, 1997), it became of interest to investigate the spatial expression of endogenous annexin V during the developmental stages in which exogenous annexin V-biotin was previously found to label apoptotic cells. The expression pattern of annexin V mRNA visualized by *in situ* hybridization

indeed showed overlap with the annexin V-biotin staining, suggesting that *annexin V* is transcribed in regions of developmental cell death (van den Eijnde, *et al.*, 1998b; S. van den Eijnde and H. Heus, unpublished results). For example, staining for annexin V mRNA was observed in E11, E12 and E13 limb mesoderm, located pre- and postaxially in the progress zone and interdigitally, respectively. In E10 and E11 embryos, also a strong staining was observed in the developing cerebellum at borders of the rhombencephalon, in the facial swellings shortly before and during fusion, and in the lens vesicle during detachment from the ectoderm. Furthermore, in E9 and E10 embryos, an intense staining was observed in the otic vesicle.

Although in the negative-control experiments, using a sense probe for annexin V mRNA, labeling was absent, these experiments still should be considered as preliminary until other annexin V probes have also been tested and have been shown to overlap with the presently obtained staining patterns. This is especially of importance because the different annexins share a strong sequence homology, and during development more members of the family may simultaneously be expressed (Ivanenkov *et al.*, 1994). Nonetheless, a specific staining for annexin V expression in these experiments is indicated by the staining of the otic vesicle in mice, which corroborates the findings with regard to *annexin V* expression in zebrafish (Farber and Halpern, 1997). Furthermore, staining for annexin V mRNA was observed in mineralizing phalanges in E14 limbs, which is in line with immunocytochemical findings previously reported by Rahman and co-workers (Rahman *et al.*, 1997). Moreover, in similar immunocytochemical experiments, using a polyclonal antibody raised against human recombinant annexin V, which cross reacts with the murine protein, we were also able to detect the endogenous protein in paraffin sections through E14 limbs (van den Eijnde *et al.*, 1998b). In addition, in E13 mouse embryos staining was observed of apoptotic cells at several locations, including limb tissue and dorsal root ganglia. Although only cells were found labeled which showed advanced chromatin condensation, most apoptotic cells were unlabeled with the antibody (S. van den Eijnde, unpublished results). Whether the high specificity but limited sensitivity of the antibody to bind apoptotic cells accurately mirrors the protein distribution of annexin V during embryogenesis remains an open question which may be answered by using anti-murine annexin V antibodies or antigen retrieval techniques. Nonetheless, the combined data of the *in situ* hybridization and immunocytochemical studies are strongly suggestive for the expression of annexin V in areas of developmental cell death, which makes it tempting to speculate about roles of this protein in these areas.

b. Speculating about the *in vivo* function(s) of annexin V

With regard to putative functions of endogenous annexin V during development, two localization patterns have to be considered: the expression of annexin V in bone anlagen, and in areas of developmental cell death (see above). The former may be related to the formation of apetite crystals and chondrocyte mineralization, which has been suggested to be related to a calcium channel activity and maybe also to the capacity of annexin V to bind collagen type II, an extracellular matrix molecule (King *et al.*, 1997; Kirch *et al.*, 1997; Rahman *et al.*, 1997). In contrast, the spatiotemporally matching of the staining of exogenous human recombinant annexin V and expression of endogenous annexin V in several areas of developmental cell death are difficult to relate to the formation of apetite crystals. Possibly, the function of annexin V in these regions can be found in the common denominators in these regions: cell differentiation and regulation of cell numbers (chapter 5). Such a role of annexin V in these regions is substantiated by observations from *in vitro* studies which showed that an increase in intracellular annexin V

concentration is related to differentiation, and the entering of the G_0-phase by neuronal cells (Schleapfer *et al.*, 1990), and to inhibition of tumor cell proliferation (Shibata *et al.*, 1997). Possibly these functions are mediated by annexin V's PLA_2 and PKC inhibitory activities; these two molecules are thought to mediate cellular differentiation and cell proliferation. In addition, through its PKC inhibitory activity, annexin V may also mediate the occurrence of apoptosis, since it has been shown that B cells that were stimulated to proliferate activate the cell death program if PKC is inhibited (Knox *et al.*, 1995).

Similar to areas of apoptosis in developing specimens, also in the immune system the modulation of cell differentiation, proliferation, and apoptosis is paramount (Wride *et al.*, 1995; Seledtsov *et al.*, 1996; Mehler *et al.*, 1997). Interfering with these processes may also explain the immunosuppressive effect of annexin V on polyclonally (LPS and anti-CD40) activated B cells *in vitro* (chapter 6). This was indicated by ^{3}H-thymidine incorporation experiments which showed that annexin V strongly impaired B cell proliferation. In addition, flowcytometric analysis with the membrane impermeable dye propidium iodide showed that the percentage of viable cells markedly decreased in the presence of annexin V. The result of both effects of annexin V is probably a net reduction of the B cell-population size, leading to a profound inhibition (~-90%) of IgG_1, IgE and IgM production. No definitive answer is yet obtained with regard to the question whether annexin V inhibits isotype production through binding a receptor at the B cell, or via an intracellular activity. However, results from confocal laser scanning experiments of B cells incubated for 24 hours with annexin V conjugated to the fluorescent marker Oregon Green, did reveal that annexin V may indeed enter the B cell (chapter 6). Interestingly, both wild type annexin V and mutant annexin V with a reduced capacity to bind Ca^{2+} and PS, *i.e.* M1 and M1234, were equally capable to inhibit isotype production. This indicates that annexin V interacted with the activated B cells in a calcium and phospholipid independent manner. When entering the cell, the immunomodulatory activity of M1234 is probably best explained by an inhibition of PKC activity, causing inhibition of B cell proliferation and leading to apoptosis (Knox and Gordon, 1995), albeit M1234 is a less potent PKC inhibitor than wild type annexin V (Mira *et al.*, 1997).

It is noteworthy, that in addition to inhibition of B cell proliferation, similar doses of annexin V inhibited T cell proliferation (K. Radosevic, P. J. Leenen, S. M. van den Eijnde, unpublished data), which may also be mediated by the inhibition of intracellular PKC (Dubois *et al.*, 1995b). Various studies have indicated that the immunomodulatory activities of annexin V are possibly even more extensive; annexin V is suggested to suppress neutrophil chemotaxis, and the release of eicosanoids and arachidonic acid (Perretti *et al.*, 1991; Becherucci *et al.*, 1993). In addition to these *in vitro* results, annexin V has been shown to inhibit interleukin 1-beta induced pyresis in rabbit too, probably by inhibiting the rise of cerebrospinal fluid levels of PGE_2 (Palmi *et al.*, 1995). These anti-inflammatory activities of annexin V were attributed to inhibiting—extracellular—$sPLA_2$ activity. This activity may be due to both a PS dependent and a PS independent interaction of annexin V with plasma membranes, as was indicated by studies using wild type annexin V (Becherucci *et al.*, 1993) or using oligopeptides derived from regions of highest similarity between annexin V, annexin I and uteroglobin (Perretti *et al.*, 1991; Palmi *et al.*, 1994; Palmi *et al.*, 1995). It may be hypothesized that annexin V, by regulating cell differentiation, cell numbers and $sPLA_2$ activity, is an important factor in maintaining homeostasis. This notion may even be further substantiated by investigations which have shown that annexin V may act as a potent anticoagulant by binding PS molecules exposed at the surface

of activated platelets and thus inhibiting the PS catalyzed conversion of prothrombin into thrombin (Andree *et al.*, 1992; van Heerde, 1994).

Future direction

To date, the roles of annexin V *in vivo* still remain to be elucidated. Instead of narrowing down the number of putative functions of annexin V, the data presented in this thesis argue for addition of the modulation of apoptosis to the list of putative functions of annexin V. Clarifying the *in vivo* functions of annexin V is a challenging field with many obstacles to be taken. Some of the obstacles may be found in the differential regulation of annexin V expression which in rat is under control of a household-gene promoter and a glucocorticoid inducible promoter (Learmonth *et al.*, 1992). Moreover, it needs to be considered that endogenous annexin V possibly does not only have a function inside the cells, but that it also may play a role outside the cells, since this protein, though lacking a signal peptide (Christmas *et al.*, 1991), was found at high concentrations in prostate fluid (approximately 60 μg/ml) and more diluted in seminal plasma (Christmas *et al.*, 1991). During pregnancy, high levels of annexin V were measured in maternal blood (up to 225 ng/ml) and in the amniotic fluid (highest level 180 ng/ml) (S. van den Eijnde, H. Brandenburg, unpublished results). The results from the latter study indicate that endogenous annexin V probably also exists in the extracellular compartment of human embryos. Interestingly, the distinct decrease in annexin V levels in the amniotic fluid after 32 weeks of gestation that were observed in the same study (highest level 70 ng/ml) may reflect the development of the glomerulus, in particular with respect to the negatively charged glomerular basement membrane (Braunwald *et al.*, 1987).

Further deciphering the roles of annexin V in normal physiology and disease is probably only possible in combined efforts. These may encompass *in vitro* studies using annexin V mutant proteins to elucidate the structure function relationship of annexin V, whereas the generation of annexin V genetic mutant mice will probably aid the insight in the *in vivo* functions of annexin V. If annexin V is a key protein in normal development and adult homeostasis, it is likely that animals defective for the protein will not be viable. Though, if studies can be conducted to obtain offspring which is devoid of annexin V during selective phases of existence, informative phenotypical changes may be observed in mouse embryos in areas of apoptosis and bone differentiation, and during placentation; in adult mice, the phenotype may possibly reflect a disturbance of tissue homeostasis. Furthermore, producing animals (over)expressing annexin V can help to elucidate the question raised in the first part of this thesis research: "what is the role of PS exposure by apoptotic cells and viable cells at specific phases of existence." In this scenario, transgenic *Drosophila* flies are probably the model of choice, because insects have no inflammatory cells, which in vertebrate systems by interacting with annexin V might markedly complicate the picture.

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Summary

Chapter 1

In chapter 1, a literature survey is presented with regard to the process of apoptosis, c.q. physiological- or programmed cell death. At the light microscopical level, the most profound characteristic of apoptosis is cell shrinkage; thereby these cells, which die in an organized manner, can clearly be discriminated from "ballooning" necrotic cells which die by accident as a part of pathology. The presence of apoptosis in developing and adult tissues is discussed in this chapter, as well as the consequences of too much or too few apoptotic cells for normal development and tissue homeostasis. For example, derailment of the normal presence or absence of apoptosis may result in a variety of congenital malformations, neurodegenerative disorders, cancer, and heart and autoimmune disease.

Most research of apoptosis presently focuses on the molecular control of apoptosis, which includes a cell type and species specific signaling phase, and subsequent phylogenetically conserved phases of apoptosis activation and execution, and phagocytosis. One of the mediators of phagocytosis is the exposure of the phospholipid phosphatidylserine (PS) at the membrane surface of the dying cells, as has recently been shown by *in vitro* studies. Finally, in this chapter the scope of the thesis is delineated, which is twofold. Firstly, it pertains the study of PS exposure by apoptotic cells *in vivo* in developing specimens, using human recombinant Annexin V, a Ca^{2+} binding protein with a high affinity for PS, as a tool (chapters 2-5). Secondly, to get insight in putative *in vivo* functions of annexin V, human protein recombinant was investigated for its immunomodulatory capacity in an *in vitro* B cell-immunomodulation assay (chapter 6), and spatiotemporal expression patterns of endogenous annexin V were documented in mouse embryos (chapter 7).

Chapter 2

By applying fluorescent annexin V to cryostat sections through mouse embryonic tissue, it is shown that all cells contain PS molecules, which can be detected if the cell is cut open. In contrast, this protein only binds specific sets of cells if presented to the cells in such a manner that it can only detect PS present in the outer plasma membrane leaflet, as occurred after intracardial injection of annexin V conjugated to biotin (annexin V-biotin). The study of the spatiotemporal distribution of annexin V-biotin in mouse embryos revealed that annexin V binding cells mainly located in regions of developmental cell death. Further evidence that these cells were apoptotic came from an ultrastructural study of annexin V-biotin labeled cells in mouse-limb tissue, showing that these cells were having the morphological characteristics of apoptosis. In addition to apoptotic cells, two cell types were observed annexin V labeled, but clearly viable, *i.e.*, fusing muscle cells, and megakaryocytes/megakaryoblasts. In the light of these results, the role of cell surface exposure of PS is discussed with regard to heterotypic cell recognition during phagocytosis of apoptotic cells, and homotypic cell recognition during myoblast and megakaryocyte differentiation.

Chapter 3

Many of the components of the molecular program of apoptosis have been shown to be conserved in evolution. By injecting annexin V-biotin into the circulation of mouse and chick embryos, and into the hemolymph of *Drosophila* pupae, it is shown in chapter 3 at the light microscopical level that mammalian, avian and insect cells are all capable to bind annexin V at the plasma membrane when apoptotic. In contrast, these cells were negative for annexin V when in the viable phase of existence. These data indicate that cell surface exposure of PS during apoptosis is probably a phylogenetically conserved component of apoptosis.

Chapter 4

The study of apoptosis requires specific and sensitive markers to detect the dying cells. In this chapter, annexin V-biotin labeling of apoptosis in mouse embryos *in vivo* is studied in whole mount embryos, and in paraffin and utrathin sections. In addition, annexin V labeling was compared with labeling of apoptotic cells in (1) whole mounts using Nile blue sulfate, and (2) in paraffin sections, after staining for apoptosis associated DNA fragmentation using the TUNEL method. The results from this study indicate that annexin V-biotin is a versatile marker for the detection of apoptotic cells *in vivo*, enabling the detection of apoptotic cells at the whole mount- to ultrastructural level. Furthermore, in comparison to the TUNEL method which mainly labels late-apoptotic/pyknotic cells, annexin V showed to be a relatively early marker of apoptosis detecting apoptotic cells preceding the pyknotic phase .

Chapter 5

Using annexin V-biotin as a marker of apoptotic cells, in chapter 5 neuronal cell death was studied (1) *in vitro* in NGF deprived neonatal rat neurons derived from dorsal root ganglia, and (2) *in vivo* in the central (CNS) and peripheral (PNS) nervous system of early mouse embryos. Both *in vitro* and *in vivo*, annexin V-biotin showed to specifically label apoptotic neurons. Of special interest is that these neurons were labeled across the whole perimeter, including somata and extensions. The labeling of apoptotic cells in the PNS was concentrated in areas of morphogenesis of the early brain, but also scattered cell death was observed, probably associated with neuronal proliferation and differentiation. In the PNS, it was shown that neurons from the large clear group were especially prone to become apoptotic during E10-13, probably mediated by the expression of limited amounts of neurotrophic factors BDNF and/or NT3 in their projection areas. Furthermore, quantification of apoptotic cells showed that approximately half of the apoptotic cells in the PNS were labeled with annexin V-biotin, whereas the other half of the apoptotic cells, recognizable by their pyknotic nuclei, were unlabeled. In line with previous observations in embryonic limbs (chapters 2 and 4), also the ultrastructural study of nervous tissue showed that unlabeled apoptotic cells were located in phagocytes. Presumably, these cells were annexin V negative because they were already present in phagocytes during perfusion with annexin V-biotin. However, in contrast to limb mesoderm which contained both unlabeled and labeled phacytosed apoptotic cells, in the nervous tissue all labeled apoptotic cells were located outside phagocytes. This suggests that phagocytic clearance of apoptotic cells differs between

nervous tissue and limb mesoderm, possibly with respect to the surface epitopes mediating phagocyte recognition of apoptotic cells.

Chapter 6

In chapters 2-5, human recombinant annexin V was used to probe for surface exposure of PS by apoptotic cells, and utilized as a marker to detect apoptotic cells. However, expression patterns of endogenous annexin V in adult tissues, and the presence of anti-annexin V antibodies in adult blood, suggest a function of endogenous annexin V in inhibition of blood coagulation and in modulating the (B cell) immune response. In chapter 6, it was tested whether annexin V was able to suppress immunoglobulins (Ig)G_1, -E and -M production by LPS and anti-CD40 stimulated B cells. The data indicate that annexin V inhibits production of these isotypes dose dependently. This Ig-production inhibiting effect of annexin V is probably mediated by the capacity of this protein to downregulate B cell proliferation and to increase B cell death. Confocal laser scanning studies suggest that annexin V is probably actively internalized by B cells. In addition, studies using mutant annexin V with a reduced or absent capacity to bind PS, and using heat inactivated annexin V, suggest that annexin V interacts with B cells in a PS independent manner, and needs an intact tertiary structure for its immunomodulatory action. Combined, the internalization experiments and structure-function studies suggest that annexin V may suppress the B cell immune response by inhibiting PKC activity.

Chapter 7

The first part of chapter 7 focuses on the use of annexin V as a tool to study cell surface exposure of PS. The commitment of apoptotic cells to expose PS during the process of apoptosis is discussed in combination with the role of this plasma membrane alteration in phagocytic clearance of apoptotic cells. Furthermore, the hallmark-nature of cell surface exposed PS by apoptotic cells, and the use of annexin V as a marker is analyzed in further detail.

In the second part of this chapter, the putative *in vivo* functions of annexin V are discussed. For annexins in general a variety of *in vitro* functions have been described, but the physiological functions of the different family members are still enigmatic. Data from expression studies of annexin V in mouse embryos, presented in this chapter, suggest a role of endogenous annexin V in areas of apoptosis and bone development during embryogenesis. Key events in these regions are differentiation, proliferation and cell death (see chapter 5), whereas similar events are paramount in the regulation of the immune system (see chapter 6). Based on these data, it is hypothesized that annexin V may have a function in maintaining homeostasis.

Samenvatting

Hoofdstuk 1

In hoofdstuk 1 wordt een literatuuronderzoek gepresenteerd met betrekking tot het proces van apoptose, c.q. fysiologische- of geprogrammeerde celdood. Op lichtmicroscopisch niveau is het condenseren van de cellen, pyknose genoemd, de meest kenmerkende celmorfologische eigenschap van apoptose; hierdoor kunnen deze cellen die georganiseerd dood gaan duidelijk onderscheiden worden van opzwellende necrotische cellen, die ongeorganiseerd dood gaan als onderdeel van een pathologisch proces. Het aanwezig zijn van apoptose in zich ontwikkelende en volwassen weefsels wordt bediscussieerd in dit hoofdstuk, tezamen met de gevolgen van een teveel of tekort aan apoptose voor de normale ontwikkeling en de weefselhomeostase. Bijvoorbeeld, disregulatie van apoptose kan leiden tot verschillende aangeboren afwijkingen, neurodegeneratieve aandoeningen, kanker, hartziekten en autoimmuunziekten. Heden ten dage is het meeste apoptose-onderzoek gewijd aan de moleculaire regulatie van dit type celdood. Uit verschillende studies is gebleken dat apoptose een meerstaps proces is, opgebouwd uit een celtype-specifieke signaalfase, en fylogenetisch geconserveerde fasen van apoptose-activatie, -executie, en -fagocytose. De laatste fase omvat ook het tot expressie brengen van het phospholipide phosphatidylserine (PS) aan het membraan oppervlak van stervende cellen, zoals recentelijk *in vitro* is aangetoond.

Tot slot wordt in dit hoofdstuk de tweeledige doelstelling van dit proefschrift omschreven. In de eerste plaats behelst het de studie van het exposeren van PS door apoptotische cellen *in vivo* in zich ontwikkelende dieren door humaan recombinant annexine V, een Ca^{2+} bindend eiwit met hoge affiniteit voor PS, te gebruiken als een gereedschap (hoofdstukken 2-5). Als tweede doelstelling heeft dit proefschrift het verkrijgen van inzicht in mogelijke *in vivo* functies van annexine V. Hiertoe werd de B cell-immuunrespons modulerende werking van humaan recombinant annexin V *in vitro* onderzocht (hoofdstuk 6), en werden de spatiotemporele expressie patronen van endogeen annexine V in kaart gebracht in muizenembryo's (hoofdstuk 7).

Hoofdstuk 2

Door het aanbrengen van fluorescerend annexine V op vriescoupes door muizenembryo's, wordt in dit hoofdstuk aangetoond dat alle cellen PS bevatten, wat gedetecteerd kan worden als de cel geopend is. Als annexine V echter zodanig aan de cellen wordt aangeboden dat het alleen PS aan de buitenkant van cellen kan bereiken, bindt het alleen aan specifieke subsets. Dit laatste kan worden aangetoond door het inspuiten van annexine V geconjugeerd aan biotine (annexine V-biotine) in het hart van levende embryo's. De studie van de spatiotemporele distributie van annexine V-biotine in muizenembryo's maakte duidelijk dat annexine V bindende cellen zich voornamelijk bevinden in gebieden waar apoptose aanwezig is tijdens de embryonale ontwikkeling. Aanvullend bewijs dat deze cellen apoptotisch waren, kwam van een electronenmicroscopische studie van weefsel van muizenextremiteiten, waarin de gelabelde cellen de morfologische kenmerken van apoptose vertoonden. Naast apoptotische cellen werden ook twee typen annexine V-gelabelde cellen aangetroffen die duidelijk vitaal waren, namelijk fuserende spiercellen, en megakaryoblasten/megakaryocyten. In het licht van deze resultaten wordt de rol

van PS expositie besproken ten aanzien van de heterotypische herkenning tijdens de fagocytose van apoptotische cellen en de homotypische herkenning tijdens de spiercel-differentiatie en de rijping van megakaryocyten.

Hoofdstuk 3

Veel componenten van het moleculaire programma van apoptose blijken fylogenetisch geconserveerd te zijn. Middels het inspuiten van annexine V-biotine in de circulatie van muizen-en kippenembryo's, en in de hemolymfe van *Drosophila* poppen wordt in hoofdstuk 3 op lichtmicroscopisch niveau aangetoond dat apoptotische cellen van een zoogdier, vogel en insekt in staat zijn om annexine V te binden, terwijl deze cellen geen annexine V binden tijdens de vitale fase van hun bestaan. Deze gegevens laten zien dat het exposeren van PS door apoptotische cellen een fylogenetisch geconserveerd onderdeel is van het apoptoseproces.

Hoofdstuk 4

Voor apoptose-onderzoek zijn markers gewenst die specifiek en gevoelig zijn. In dit hoofdstuk is de annexine V-biotine labeling van apoptotische cellen *in vivo* in muizenembryo's bestudeerd, zowel in hele embryo's, als in paraffine en ultradunne coupes. De annexine V labeling van apoptotische cellen werd vergeleken met de labeling van apoptotische cellen in (1) hele embryo's met behulp van een Nijl blauw sulfaat kleuring, en (2) in paraffine coupes na kleuring voor apoptose geassocieerde DNA fragmentatie middels de TUNEL methode. De resultaten van deze studie geven aan dat annexine V een veelzijdige marker is waarmee apoptotische cellen aangetoond kunnen worden zowel in hele embryo's als op elektronen microscopisch niveau. Bovendien is annexine V-biotine een vroege marker van apoptose die cellen detecteert al voor deze de pyknotische fase bereikt hebben. Dit in tegenstelling tot de TUNEL methode waarmee voornamelijk pyknotische apoptotische cellen gelabeld worden.

Hoofdstuk 5

Door gebruik te maken van annexine V-biotine als marker, is in hoofdstuk 5 neuronale celdood bestudeerd (1) *in vitro* in ganglioncellen van de achterhoorn waaraan NGF onthouden was, en (2) in het centrale (CZS) en perifere (PZS) zenuwstelsel van vroege muizenembryo's. Zowel *in vitro* als *in vivo* bleek dat annexine V-biotine specifiek apoptotische neuronen aankleurt. Van bijzonder belang is dat deze kleuring aanwezig was aan de gehele buitenkant van de cel, inclusief het cellichaam en de uitlopers. De labeling van apoptotische cellen in het CZS concentreerde zich in gebieden van morfogenese van de embryonale hersenen. Ook verspreide celdood werd aangetroffen. Deze is mogelijk gerelateerd aan neuronale celproliferatie en celdifferentiatie. In het PNS werd aangetoond dat specifiek cellen van de grote-heldere groep sterven op dag 10-13 van de embryonale ontwikkeling. Deze celdood wordt mogelijk gemedieëerd door de aanwezigheid van beperkte hoeveelheden van de neurotrofe factoren BDNF en NT3 in de projectiegebieden van deze neuronen. Door de apoptotische cellen in het PZS kwantificeren, bleek dat ongeveer de helft van de apoptotische cellen gelabeld wordt met annexin V-biotine, terwijl de andere helft van de apoptotische cellen, herkenbaar aan de pyknotische kern, ongelabeld bleek te zijn. In overeenstemming met waarnemingen in de extremiteiten van muizenembryo's (hoofdstukken 2 en

4) toonde ook electronenmicroscopisch onderzoek van neuronaal weefsel aan dat ongelabelde cellen in fagolysosomen aanwezig waren. Waarschijnlijk waren deze cellen reeds gefagocyteerd voordat annexine V was toegediend. Een verschil tussen het pootknop-weefsel en neuronale weefsel was echter dat in het eerste weefsel naast ongelabelde apoptotische cellen ook frequent gefagocyteerde gelabelde cellen werden aangetroffen, terwijl in het neuronale weefsel alle gelabelde cellen zich buiten de fagocyten bevonden. Dit suggereert dat het verwijderen van apoptotische cellen verschilt tussen pootknop-mesoderm en neuronaal weefsel. Dit is mogelijk gerelateerd aan de oppervlakte epitopen die dit proces reguleren.

Hoofdstuk 6

In de hoofdstukken 2-5 werd humaan recombinant annexine V gebruikt om de expressie van PS aan het oppervlak van apoptotische cellen aan te tonen, en werd het toegepast als apoptose marker. Echter, het expressie-patroon van endogeen annexine V in volwassen weefsels, en de aanwezigheid van antistoffen tegen annexine V in het bloed van volwassenen suggereert een functie van endogeen annexine V in de remming van de bloedstolling en de modulatie van de (B cel) immuunresponse. In dit hoofdstuk is getest of annexine V de produktie van de immunoglobulinen (Ig)G_1, -E en -M door LPS en anti-CD40 gestimuleerde B cellen kan remmen. De resultaten tonen dat annexine V dosis afhankelijk de produktie van de verschillende Ig-isotypen kan remmen. Dit effect van annexine V is waarschijnlijk het gevolg van het vermogen van dit eiwit om de B-cel proliferatie te remmen, en B-celdood te stimuleren. Annexine V internalisatie studies met de confocale laser scanner in combinatie met structuur-functie studies met mutant en hitte geïnactiveerd annexine V suggereren dat annexine V de B-cel immuunrespons mogelijk moduleert door inhibitie van PKC activiteit.

Hoofdstuk 7

Het eerste deel van hoofdstuk 7 is gericht op het gebruik van annexine V als een gereedschap om de expositie van PS aan het celoppervlak te bestuderen, met name van apoptotische cellen. De plasmamembraanverandering die plaatsvindt tijdens apoptose wordt bediscussieerd in relatie tot het proces van fagocytose. Tevens wordt in dit hoofdstuk besproken in welke mate PS expositie het predikaat "algemeen kenmerk van apoptose" mag dragen, en komt de bruikbaarheid van annexine V als apoptosemarker aan de orde.

In het tweede deel van dit hoofdstuk worden mogelijke *in vivo* functies van annexine V besproken. Een variëteit van *in vitro* functies is in de loop van de tijd toegeschreven aan annexine V, maar de fysiologische functies zijn nog niet opgehelderd. Gegevens afkomstig van expressie-studies van annexine V in muizenembryo's die in dit hoofdstuk worden beschreven, suggereren een rol van het endogene eiwit in gebieden van apoptose en botdifferentiatie. Sleutelprocessen in deze gebieden zijn differentiatie, proliferatie en celdood (zie hoofdstuk 5), terwijl dezelfde processen ook van groot belang zijn voor de regulatie van het immuunsysteem (zie hoofdstuk 6). Op grond van deze gegevens is de hypothese opgesteld dat annexine V mogelijk een functie vervult in het behoud van de homeostase. Tot slot worden suggesties gedaan voor verdere experimenten met betrekking tot het in kaart brengen van de fysiologische functie(s) van annexine V.

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Curriculum Vitae

De schrijver van dit proefschrift werd op 17 januari 1967 geboren te Velsen. Hij is opgegroeid in Haarlem, waar hij de lagere en middelbare school heeft gevolgd (Spaarne Lyceum). In 1986 startte hij de studie Biomedische Wetenschappen aan de Rijksuniversiteit Leiden en behaalde het het doctoraalexamen op 24 augustus 1992. Vanaf januari 1993 tot en met december 1997 is hij in dienst geweest bij de afdeling Klinische Genetica van de Erasmus Universiteit Rotterdam (hoofd: prof. dr H. Galjaard). Het eerste jaar van de aanstelling stond in het kader van het project "Genen verantwoordelijk voor cleft-palate", onder leiding van dr P. Demant (Afd. Moleculaire Genetica, Nederlands Kanker Instituut, Amsterdam) en mw. dr Chr. Vermeij-Keers (Afd. Anatomie en Inst. Plastische Heelkunde, Erasmus Universiteit Rotterdam). In de daaropvolgende periode, van januari 1994 tot en met december 1997 verrichtte hij binnen de groep van mw. dr Chr. Vermeij-Keers het onderzoek dat ten grondslag ligt aan dit proefschrift. In deze jaren is hij in het huwelijk getreden met A. J. Y. Hofland en vader geworden van S. Q. van den Eijnde. Geheel 1998 is hij in dienst geweest van het Instituut Plastische Heelkunde (hoofd: prof. dr S. E. R. Hovius), en werkte hij in nauwe samenwerking met de groep van dr C. P. M. Reutelingsperger (Afd. Biochemie, Universiteit Maastricht) aan een *in vivo* model ter bestudering van fagocytose. Op 1 januari 1999 is hij als post-doc gestart bij de afdeling Moleculaire Celbiologie en Genetica van de Universiteit Maastricht (hoofd: prof. dr F. C. M. Ramaekers), waar hij onderzoek verricht binnen het project "Moleculaire ontwikkelingsbiologie van het myocard".

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Ik heb het onderzoek voor een groot deel uitgevoerd als gast binnen de Afdeling Anatomie van de Erasmus Universiteit Rotterdam. Na afloop van mijn aanstelling bij het Instituut Klinische Genetica heb ik mijn werk op dezelfde verdieping voortgezet binnen het Instituut Plastische Heelkunde. Ik wil prof. dr J. Voogd en respectievelijk prof. dr S. E. R. Hovius hartelijk danken voor hun gastvrijheid en het ondersteunen van mijn onderzoek. Professor Voogd wil ik bovendien bedanken voor het pre-viewen van een aantal van mijn publikaties.

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Dr Eric Baehrecke, dear Eric, I see you as a good friend, and you are an example, both as a person and as a scientist. I find it inspiring to see how you promote your favorite model system,

Drosophila, and the way you combine molecular research and a detailed know how of *Drosophila* development.

Dr Jan Hein van Dierendonk, we hebben heel wat afgediscussieerd over apoptose, en we hebben tot in de vroege uren gewerkt aan een dubbel-labeling-experiment voor DNA-fragmentatie en annexine V. Het zijn mooie herinneringen, en wat mij betreft mogen er daarvan nog heel wat bijkomen.

Aan het eind van het promotieonderzoek, op zoek naar mogelijke functies van annexine V, viel mijn oog op de immunologie. Van de Afdeling Immunologie van de EUR wil ik dr Katarina Radocevic en dr Pieter Leenen hartelijk bedanken voor het maken van de vertaalslag van vraagstelling tot experiment. Dr Huub Savelkoul en dr Adrie van Oudenaren, bedankt voor de tijd die jullie aan deze studie hebben gewijd. Adrie, samen hebben we hard gewerkt aan het artikel. Ik ben onder de indruk van jouw geduld, zeker op momenten dat ik weer eens aan het doordraven was.

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Samengevat: collega's/medewerkers van de afdelingen/instuten Anatomie, Immunologie, Celbiologie en Genetica, Gynaecologie en Verloskunde, Klinische Genetica, Pathologie en Plastische Heelkunde van de Erasmus Universiteit Rotterdam, Afdeling Biochemie en Afdeling Moleculaire Celbiologie en Genetica van de Universiteit Maastricht, Afdeling Fysiologie en Afdeling Heelkunde van de Universiteit Leiden en het University of Maryland Biotechnological Institute, bedankt!

Wat de afgelopen jaren een prominente plaats heeft ingenomen in mijn leven staat aan het begin van dit proefschrift, apoptose en annexine V. Hier, zo een honderd pagina's later is het moment om de wetenschap weer in perspectief te plaatsen en te zien waar het werkelijk om draait: mensen, vrienden, familie en in het bijzonder mijn gezin. Annemarie en Sanne, vanavond ben ik vroeg thuis...