CELLULAR AND IMMUNOLOGICAL ASPECTS OF BASAL CELL CARCINOMA

CELLULAIRE EN IMMUNOLOGISCHE ASPEKTEN VAN HET BASAALCELCARCINOOM

The cover represents a schematic illustration of a tumor nest attacked by immunocytes, which are "trapped" and "inactivated" by the tumor cells (designed by T. van den Broek).

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"All kids are a lifetime investment"
Arnold Schwarzenegger

Voor Pa en Ma
Voor Jeroen

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LIST OF ABBREVIATIONS

List of abbreviations

APC	Antigen-presenting cell
BCC	Basal cell carcinoma
BPAG	Bullous pemphigoid antigen
BSA	Bovine serum albumin
BM	Basement membrane
Ck	Cytokeratin
CTL	Cytotoxic T lymphocytes
DAB	3,3'-diaminobenzidine
DRC	DNA repair capacity
EC	Endothelial cells
ECM	Extracellular matrix
FA-2	Fetal antigen-2
GAM	Goat-anti-mouse
H&E	Hematoxylin and eosin
H&N	Head and neck
ICAM-1	Intercellular adhesion molecule-1
IEM	Immunoelectron microscopy
IFN	Interferon
IFN-γR	IFN-γ receptor
IL	Interleukin
IMDM	Iscove's modified dulbecco's medium
IU	International units
LAK	Lymphokine activated killer
LC	Langerhans cell
LM	Light microscopy
LOH	Loss of heterozygosity
MAb	Monoclonal antibody
MAGE-1	Melanoma antigen-1
MHC	Major histocompatibility complex
MM	Malignant melanoma
MMP	Matrix metalloprotease
MMS	Mohs' micrographic surgery
MWUT	Mann Whitney U test
NBCCS	Nevoid basal cell carcinoma syndrome
NGS	Normal goat serum

NHS	Normal human serum
NK	Natural killer
NMSC	Non-melanoma skin cancer
PA	Plasminogen activator
PAb	Polyclonal antibody
PB	Peripheral blood
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PTCH	Patched gene
rHuIFN-γ	Recombinant human IFN-γ
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficiency
Shh	Sonic hedgehog
sICAM-1	Soluble ICAM-1
SMO	Smoothened
SRCT	Spearman's rank correlation test
TAA	Tumor-associated antigen
TCR	T cell receptor
TGF	Transforming growth factor
Th	Helper T lymphocyte
TIMP	Tissue inhibitor of MMP
TNF	Tumor necrosis factor
UVR	Ultraviolet irradiation
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA	Very late activation antigen
WT	Wilcoxon test for paired observations

Chapter 1

GENERAL INTRODUCTION

1.1. Cancer of the skin.

The skin, which is the largest organ of the human body, has several important functions such as protection against infections and chemical and physical influences, regulation of temperature and water balance, vitamin D synthesis, etc. The skin consists of two layers, the dermis and the epidermis. The dermis contains fibroblasts, mast cells, macrophages and lymphocytes, blood and lymph vessels and skin adnexa such as hair follicles with sebaceous glands and arrector pili muscles, and sweat glands. The epidermis comprises four cell types: keratinocytes, melanocytes, Langerhans cells (LC) and Merkel cells. Keratinocytes are the major cell type and they play an important role in the protection against the external environment. They differentiate from the stratum basale towards the surface of the epidermis through the stratum spinosum, the stratum granulosum and finally the stratum corneum. Melanocytes are located in the stratum basale and their main function is the production of melanin. They also play a role in the detoxification of highly reactive molecules resulting from exposure to sunlight and other sources of UV irradiation (UVR). Epidermal LC are dendritic cells, which play a role in the skin immunology by presentation of antigens to T cells. Merkel cells represent neuroendocrine cells in the epidermis and hair follicles. Tumors from all these different cell types may develop as a result of mutations, either "spontaneously" or as a consequence of exposure to chemical, physical or viral carcinogens. Examples of physical carcinogens are X-rays, radioactivity and UVR. These carcinogens cause direct DNA damage, leading to mutations, chromosomal breaks and abnormal rearrangements [1].

Skin cancer, i.e. the sum of all malignant tumors of the epidermis and the dermis, can be regarded as a progressive "silent epidemic" of the present century. In 1990, it comprised one third of all cancers in the United States [2] and its incidence is still increasing throughout the world [3-8]. Therefore, considerable effort is being made worldwide to elucidate the etiology and pathophysiology and to improve clinical management of skin cancer. The most important and most common tumors of the skin are divided into malignant melanoma (MM), derived from melanocytes, and non-melanoma skin cancer (NMSC), of which the keratinocyte-derived tumors are far the most common ones. NMSC include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), and a smaller group consisting of premalignant lesions such as Bowen's disease and keratosis actinica. Of all skin cancers, the majority (±77%) is BCC, ±20% is SCC and the remainders are MM and a small group of rare tumors, such as fibro-epithelial tumors (Pinkus).

1.2. Basal cell carcinoma

Clinical and histopathological appearances

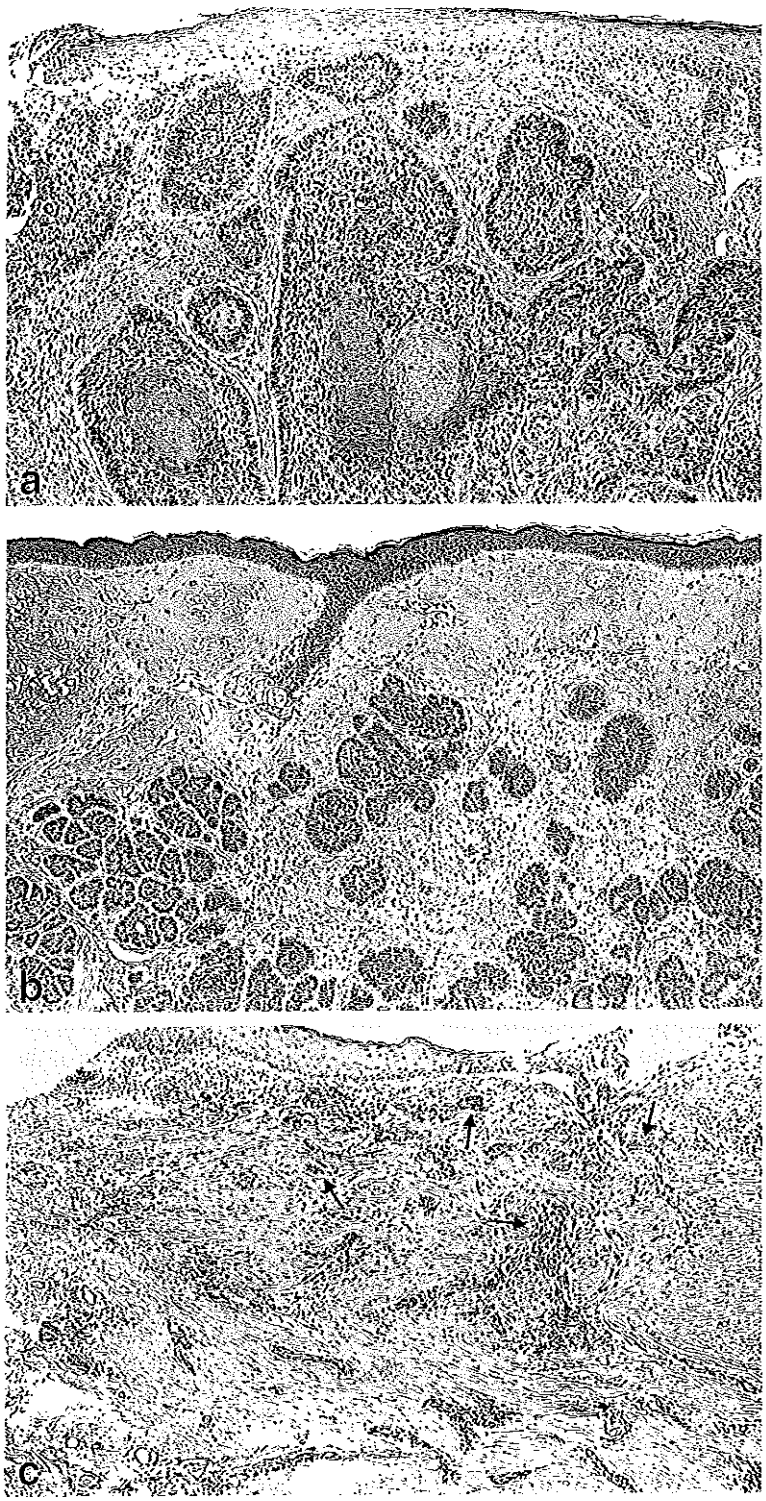
BCC is a malignant epithelial tumor, which grows invasively and may lead to extensive local tissue destruction, but it metastasizes in less than 0.1% of the cases. Like SCC, it is predominantly seen in the fair-skinned population at the mean age of 65 years [9], but has also been noted in black patients [10-13]. In 1900, Krompecher [14], was the first to describe BCC and he speculated that it originated from the basal layer of the epidermis or from skin adnexa. Since then, much research into the clinical and histopathological characteristics, origin, genetics, risk factors, therapy and immunology has been undertaken.

Different types of BCC have been described and the purpose of a proper classification of this tumor is that clinicians and pathologists are able to relate the types of the tumor to its behavior. In a recent review, discussing the histological classification of BCC, the main histopathological types of BCC recognized are: nodular, including micronodular; superficial, apparently multifocal; and infiltrative, including the morpheic type [15]. The nodular type (±50%) of BCC is characterized by rounded masses of tumor cells in the dermis, which show peripheral palisading of the nuclei. This type includes the micronodular type (±15%), which is characterized by numerous very small noduli (<0.15 mm in diameter). The superficial type (±15%) of BCC shows small buds of tumor cells, which grow down from the epidermis into the superficial dermis [15]. This type occurs more commonly on the trunk [9]. The infiltrative type (±10-20%) shows groups of tumor cells of varying size with an irregular outline and spiky projections. The peripheral palisading of the nuclei is either absent or poorly developed. This type includes the morpheic type (±5%) which is characterized by small, irregular groups or cords of tumor cells. Finally, mixed types of BCC (±10-15%) showing a combination of the above-mentioned growth patterns may occur [15].

Nodular and superficial type of BCC are very often referred to as non-aggressive types; in contrast, the infiltrative type is referred to as an aggressive type of BCC. This subdivision is mainly based on the local growth characteristics of the tumor [16], metastases of BCC being vanishingly rare. However, several cases of metastatic BCC have been described [17-19]. The primary tumor in most of these studies was of the morpheic (aggressive) type and these were often extremely large (>20 cm^2). The majority of the metastases were found in the lymph nodes and the lungs. BCC have a tendency to recur after therapy. The recurrence rate depends on both the histopathological appearance, proliferation [20] and the type of treatment used. Incompletely excised tumors have a recurrence rate of ±33-39%, whereas "completely" removed tumors show recurrences in only ±1%. Tumors of

the morpheic type have the highest recurrence rate [21]. However, the superficial type frequently shows positive margins after surgery. Examples of the nodular, micronodular, infiltrative and superficial type of BCC are shown in Figure 1.
Tumors with clinical and histological features of both BCC and SCC also occur. These are known as basosquamous or metatypical carcinomas [22,23]. Histologically, there are intermediate areas of differentiation, areas of squamous differentiation, and areas that resemble a BCC with focal keratinisation. Clinically, they grow as fast as SCC (faster than BCC) and they also have their metastatic potential.
BCC grow very slow and many speculations concerning this growth pattern have been made. The cell cycle of BCC cells is about 200 hours, which is comparable with that of normal cells. However, the S-phase in BCC cells is twice as long as that in normal epidermal cells because of prolonged DNA replication [21,24]. In addition, only some of the BCC cells replicate. Grimwood et al [25] showed that proliferating cells of nodular BCC are predominantly located at the periphery of the tumor nests. This finding was corroborated in other studies using proliferation markers such as BrdU incorporation [26], antibodies against proliferating cell nuclear antigen [27] and the Ki67-antigen [24,28]. However, heterogeneous expression of Ki67 was also reported [28,29]. This low amount of proliferating tumor cells and the prolonged DNA replication may explain the slow growth rate of BCC.

Incidence and risk factors
In the United Kingdom, a roughly 2.5 fold increase in the incidence of NMSC over a 14-year period (1978-1991) was reported [3]. An increased incidence in NMSC, particularly BCC, was also reported in the Netherlands [4], other European countries [5-7], Australia [8], the United States [2] and British Columbia in Canada [30]. The male:female ratio is approximately 1:1 [3], although there are reports that men are more at risk than women [21,23,31] Individuals who develop skin cancer often have evidence of chronic sunlight exposure such as collagenosis, irregular pigmentation, wrinkling, telangiectasis of the skin and histological signs of dyselastosis, basophilic collagen degeneration or solar keratosis on sites exposed to sunlight [23,32], indicating that chronic excessive exposure to UVR of sunlight [2,32,33] is an important risk factor for NMSC. UV light can be subdivided into UVC (200-280 nm), which does not reach the earth, UVB (280-315 nm) and UVA (315-400 nm). A high dose of UVB may result in DNA protein damage. The resultant damage in certain genes (proto-oncogenes and tumor suppressor genes) may lead to, or contribute to, tumor formation. The diminishing stratospheric ozone layer allows more UVR to reach the earth and thus may play a role in the recently increased incidence of NMSC [2].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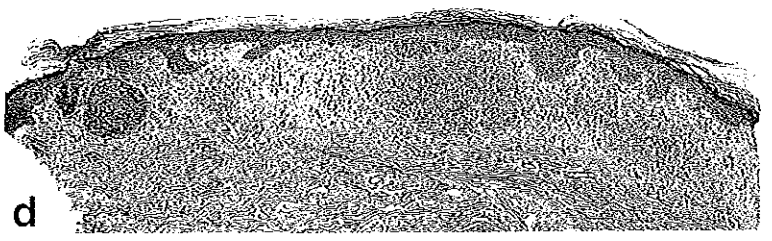

Figure 1: Hematoxylin and eosin (H&E)-stained paraffin-embedded sections of basal cell carcinoma; a, Nodular type; b, Micronodular type; c, Infiltrative type (arrows indicate the tumor cells); and d, Superficial type.

UVA is not absorbed by proteins and DNA, but can penetrate to the dermis where it has a direct damaging effect on the matrix. Besides this direct effect on epidermal cells and extracellular matrix (ECM), UVR also has an effect on the immune response. After exposure to UVR, LC were morphologically altered and reduced in number and had a reduced antigen-presentation capacity [34]. This leads to a local and, in cases of high dose of UVR, even a systemic immune suppression [35]. The production of suppressive mediators such as prostaglandin [36] and interleukin (IL)-10 [37] due to UVBR may also play an important role in the immune suppression. This total effect of UVR facilitates tumor outgrowth [38]. Thus, UVR may affect carcinogenesis through both direct epidermal changes and immune-mediated mechanisms.

It was shown that patients with SCC and those with BCC have different histories of sunlight exposure and therefore these tumors may have differences in their etiology. Development of BCC correlates with recreational sunlight exposure in childhood, especially with the number of sunburns in the first two decades of life [6,33], combined with individual factors, such as skin type and hair- and eye color [32,33]. Individuals who always burn but never tan have a higher incidence rate of BCC than those who always tan but never burn [6,8]. In contrast, SCC was shown to correlate with cumulative sunlight exposure during the entire lifetime [6,7].

The DNA damage caused by UVR is normally subject to repair by DNA repair mechanisms [39]. Patients with Xeroderma Pigmentosum, an inherited DNA repair disorder, have a more than 2000 fold higher incidence of sunlight-related skin cancer [40]. Therefore, investigations into the DNA repair capacity (DRC) in skin cancer patients were initiated. A lower DRC was observed in patients with BCC compared with controls [32].

Another important risk factor in the development of NMSC is immunosuppression. UVR has been shown to be immunosuppressive, which may inhibit selected cell-mediated immune responses directed against UVR-induced tumors [41]. Immunosuppressive therapy in organ transplant recipients appears to enhance the risk of skin cancer [42,43]. It was reported that a relatively high percentage of the patients developed SCC and/or BCC within 2-3 years after heart transplantation [42]. Similarly, in renal transplant recipients, an increased risk for the development of NMSC has also been reported, largely concerning SCC, followed by BCC. Immunosuppressive therapy and UVR are believed to be the most important risk factors in these patients [44,45]. Some of the other, minor, risk factors for BCC development are: burn scars, ionizing radiation, and prior skin cancer. [2,46-48].

Genetics

Cancer arises from cells that escape normal growth regulation through a process involving activation of growth-promoting oncogenes and inactivation of tumor suppressor genes.

An example of a proto-oncogene is the *ras* gene, which stimulates proliferation after activation. Three members of the *ras* gene family, H-*ras*, K-*ras* and N-*ras*, play an important role in the regulation of cell growth and proliferation. The highly homologous genes encode for 21 kD proteins with intrinsic GTPase activity. Point mutations in different codons of these genes convert them into active oncogenes, which have been identified in a wide variety of cancers [49]. The majority of the mutations, irrespective of the gene, are located in the codons for amino acid 12, 13 or 61 [49]. Several studies on *ras* oncogenes in BCC have been reported [26,50-54]. The gene product $p21^{ras}$ was not detected in any of the BCCs examined [26,52]. Although, point mutations in codon 12 were reported in a minority of BCC in three early studies [51,53,54], Campbell et al [50], more recently, failed to detect a mutation in codon 12 in any of the 30 BCCs that were examined. Apparently, if mutational activation of *ras* genes occurs at all, it is not a common feature in BCC.

The proto-oncogene Bcl-2 encodes for a protein that inhibits programmed cell death (apoptosis) by binding to Bax [55]. Intracellular expression of Bcl-2 in almost all BCCs was reported in several studies [28,55-61], which may point to inhibition of apoptosis in these tumors. In two studies [28,59] a decreased expression of Bcl-2 was noted in aggressive types of BCC.

The p53 gene, which is located on chromosome 17p, is known to be a tumor suppressor gene. Its gene product, a 53 kD phosphoprotein, plays a role in the regulation of the cell growth cycle [62]. Increased levels of p53 protein block cells in the G1/S stage of the cell cycle after UV-exposure, allowing DNA repair to occur prior to replication. In addition,

wild type p53 functions as a transcription factor of Bax, which induces apoptosis of cells with DNA damage [55]. Therefore, mutation of p53 results in inappropriate replication of damaged DNA and abolishes the apoptotic pathway, thereby enhancing tumor progression. Generally, normal levels of the wild type p53 gene product are too low to be detected immunohistochemically, whereas mutated p53 or increased levels of wild type p53 can often be detected. Polymerase Chain Reaction (PCR) analysis has shown, however, that immunohistochemically investigated expression may give false positive or false negative information concerning mutation of p53 [16,63,64]. In normal epidermis, chronically exposed to sunlight, significantly higher expression levels of p53 were detected compared with normal epidermis rarely exposed to sunlight. This could, however, either have been due to an increased level of wild type p53 or to an increased prevalence of mutations in the p53 gene [65]. Mutated p53 has been detected in many common tumors, including skin cancers [16,63,66-69]. Using both PCR and immunohistochemistry, p53 mutations were detected in different percentages of BCC [16,64,70,71]. Recently, using DNA sequencing, p53 mutations were detected in all 11 BCCs examined [72].

BCCs occur predominantly as sporadic cases. However, familial cases such as those in Nevoid Basal Cell Carcinoma Syndrome (NBCCS) are also encountered. NBCCS predisposes to multiple BCCs during the entire lifetime. The gene involved in this syndrome has been mapped to the 9q22-31 region [73,74]. This finding led to investigations into this chromosome in sporadic BCC. Indeed, loss of heterozygosity (LOH) of 9q22-31 was shown in these tumors. This indicates that a tumor suppressor gene in this locus in both sporadic BCC and in NBCCS is inactivated. Recently, a gene was cloned which may be the suppressor gene. This candidate gene appeared to be the human homologue of a Drosophila developmental gene; patched (PTCH) [77,78]. PTCH is part of the hedgehog-signaling pathway, which is important in determining the embryonic pattern and cell fate in multiple structures of the developing embryo [76]. The PTCH gene encodes for a 12 transmembrane domain protein that physically binds to sonic hedgehog (Shh) with high affinity [79]. In addition, smoothened (SMO), a 7 transmembrane domain protein, of which the gene is located on chromosome 7, plays a role as signaling component of the Shh receptor complex. Transgenic mice constitutively expressing Shh in the skin develop BCC-like tumors. In addition, human epidermis, transgenic for Shh, transplanted on nude mice also showed BCC features [80]. BCCs consistently show activation of the hedgehog-signaling pathway, as judged by increased PTCH mRNA [81]. These findings indicate that the hedgehog-signaling pathway plays a role in BCC development. This activation can be caused by missense mutations in SMO, which have indeed been identified in BCC [76,81,82].

In conclusion, it can be stated that inactivation or mutation of multiple suppressor genes, instead of proto-oncogenes, plays an important role in the development of BCC.

1.3. Expression patterns of cytokeratins

General expression

The cytoskeleton of the cell is a complex network of protein filaments in the cytoplasm that serves as the "scaffolding" of the cell. It plays a role in the maintaining of the shape, the internal organization and in the movement of the cell. It consists of three different types of filaments, namely the microfilaments, actin, which have a diameter of 7 nm; microtubules with a diameter of 25 nm, and intermediate filaments with a diameter of 8 to 10 nm. These intermediate filaments are composed of polypeptides that vary between different cell types [83]. At least five different types can be distinguished, namely neurofilaments, which are typical for neuronal cells; vimentin filaments which occur in mesenchymal cells, astrocytes and in many cultured cells; desmin filaments which occur in most types of muscle cells; glial filaments which are typical for astrocytes; and keratin filaments, cytokeratins (Cks), which are most common in epithelial cells [84]. However, non-epithelial human tissues, such as smooth muscle cells, have also been reported to express low levels of Cks [85].

In 1982, Moll et al [84] catalogued 19 different human Cks on the basis of biochemical and immunological criteria. Several years later an additional Ck, Ck 20, was described by these authors [86]. The isoelectric pH of these Cks varies from 5 to 8 and their molecular mass from 40 kD to 68 kD. They can be subdivided into type I (small, acidic, including Ck 9 to Ck 20) and type II (large, basic, including Ck 1 to Ck 8) Cks and they are usually coexpressed in specific pairs formed by one member of each subfamily to form a filament [85,87].

In the epidermis the expression of Cks in the basal layer differs from that in the suprabasal layer. The Ck pattern changes during wound healing [87,88] and may be influenced by cytokines such as interferon (IFN)-γ [89]. This indicates that epithelial cells can change their Ck profile during differentiation, proliferation and carcinogenesis.

Cytokeratin expression in BCC

Investigations into the expression of Cks in BCC have been pursued for several reasons. One was to establish the exact origin of BCC, because this still remains unclear. The current idea is that BCC may arise either from epidermal or from adnexal epithelium. The tissue of origin may vary between different histopathological types of the tumor.

Carcinomas tend to maintain the expression pattern of Cks typical of the epithelium they have arisen in [84]. Therefore, the Ck profile of BCC cells may point to the cell type of origin [84]. Another reason for investigating Ck patterns in BCC is to find a specific tumor marker or a suitable histopathological marker which may be used for the detection of residual tumor cells after excision or in Mohs' micrographic surgery (MMS) [92,93]. Table II shows the expression of Cks in BCC as reported in the literature.

Table I: Distribution of the different Cks in normal tissue [84,85,86,90,91].

Type of Ck	Type of tissue	Example of organ-associated tissue
Type II, large, neutral to basic		
1 and 2	Stratified epithelia	Epidermis
3	Corneal epithelia	Cornea
4-6	Non-keratinized stratified epithelia	Tongue mucosa
	Keratinized squamous epithelia	Epidermis, hair follicles
	Epithelia of trachea	
	Apocrine glands	
7 and 8	Diverse simple epithelia	Sweat glands, mammary glands
	Transitional epithelia	Urothelium
Type I, small, acidic		
9-11	Stratified epithelia	Epidermis
12	Corneal epithelia	Cornea
13	Non-keratinized stratified squamous epithelia	Tongue mucosa
14-17	Stratified epithelia	Epidermis
	Non-keratinized stratified epithelia	Tongue mucosa
18	Simple epithelia	Sweat glands
19	Simple epithelia	Sweat glands
20		Merkel cells, urothelium

Table II: Expression of Cks in BCC

Type of Ck	Expression	Reference
5	++	[84,94-98]
7	(+)	[95]
	-	[99,100]
8	+	[98,101]
	(+)	[84,97]
10	(+)	[102]
	-	[95-100]
14	++	[84,94-98]
17	++	[84,94,95,97,98]
18	(+)	[101]
	-	[95,98,100]
19	+	[95,96,98,101]
	-	[103]
20	-	[104]

- = No expression; (+) = Expression in only a few tumor cells; + = Expression in only a percentage of BCC; ++ = Strong expression in all BCC.

1.4. Immunological mechanisms in tumors in general and in basal cell carcinoma

Tumor immunology

Several mechanisms are known to induce anti-tumor immune responses. Tumor-associated antigens (TAA) play an important role in anti-tumor immunity. These antigens are (glyco)proteins that are either not expressed by normal cells; or in much lower quantities; or in a sequestered site; or qualitative alteration in protein due to mutation of the corresponding gene [105,106]. Therefore, TAA may act as targets for effectors of natural immunity. The best-known tumor antigens are melanoma antigen-1 (MAGE-1) in MM and proto-oncogenes such as p21 and p53 in a variety of tumors [106]. TAA are usually intracellular molecules, that are broken down intracellularly, resulting in fragments, which are presented in association with major histocompatibility complex (MHC) class I molecules [105]. Different immunocytes are involved in the immunity against tumors. Peripheral blood (PB) comprises 75% T lymphocytes, 10% B

lymphocytes and 15% natural killer (NK) cells. The T lymphocytes can be divided into CD4+ helper T cells (Th) (70%) and CD8+ suppressor/cytotoxic T cells (CTL) (25%). The latter play an effector role in the cell-mediated cytotoxicity against malignant cells expressing peptides presented by MHC class I molecules, whereas the Th cells may provide the necessary cytokines. Cytokines are intercellular signaling proteins that regulate local and systemic immune and inflammatory responses, as well as many other biologic processes [107]. Th cells can be subdivided into 3 categories, Th1, Th2, and Th0, based on the production of the cytokines [108]. Cytokines such as tumor necrosis factor (TNF)-α and IFN-γ, produced by Th1 cells, may induce an effective CTL development. IFNs were discovered in 1957 as soluble factors interfering with viral replication. They have the ability to regulate specific gene expression and metabolic activity in their target cells, leading to antiproliferative and immunomodulatory effects. Two types of IFN have been identified: type I includes IFN-α, -β and -ω, and type II includes IFN-γ. Both types of IFN have specific receptors, which are expressed on nearly all cell types. Binding of type I IFN to the receptor leads to up-regulation of MHC class I expression, which in virally infected cells enhances the viral antigen presentation. It may also lead to growth arrest of the cell, but not to killing. IFN-α and -ω are produced by leukocytes, whereas IFN-β is produced by fibroblasts and most other non-leukocytic cells. Only small amounts of IFN-β are produced by leukocytes. IFN-γ is an immunoregulatory cytokine, which is secreted by activated T cells and some NK cells. Binding of IFN-γ to its receptor leads to up-regulation of MHC class I and MHC class II and it may induce de novo MHC class II expression. It activates macrophages to produce IL-1, IL-6, IL-8 and TNF-α and enhances the Th1 activity, leading to up-regulation of the cell-mediated immunity [107].

In addition to T lymphocytes, NK cells are also major contributors to the immune response against tumors [109]. They kill their targets in a MHC-unrestricted manner, although they do appear to have a degree of specificity. The tumoricidal capacity of NK cells is increased by cytokines such as IFN, TNF, IL-2 and IL-12. Macrophages secrete TNF-α, which may be directly toxic for the tumor cells or may enhance CTL activity [110]. CTLs use two independent pathways to lyse their target cells, namely by exocytosis of perforin and granzyme B, both cytotoxic effector molecules, or by cell-cell interaction with the target cells [109].

Despite the presence of constant immune surveillance, it is clear that many benign and malignant tumors manage to grow and develop. Several mechanisms may enable tumors to escape this immune surveillance. These mechanisms are: 1) Down-regulation of MHC class I antigens on the tumor cells, which prevents antigen presentation and thereby recognition by the CTLs; 2) The absence of MHC class II antigens on the tumor cells, which prevents activation of tumor-specific $CD4^+$ T cells; 3) The absence of

costimulatory molecules such as intercellular adhesion molecule (ICAM)-1, CD40 and B7.1 (CD80) [109,111] on the tumor cells. This may cause T cell anergy in tumor-specific T cells, and 4) The production of immunosuppressive cytokines such as transforming growth factor (TGF)-α, IL-10 and vascular endothelial growth factor (VEGF), which may inhibit the local immune response [109,110].

Immunological mechanisms in basal cell carcinoma

Two decades ago, Dellon et al [112] was the first to investigate PB of BCC patients and these authors claimed that these patients had a significantly lower number of T cells in their PB compared with controls. This T cell count correlated with the tumor size and its reduction was transient in patients with small (< 2 cm^2) BCC. About 10 years ago, Myskowski et al observed a significantly reduced lymphocyte proliferation response to a non-specific mitogen, concanavolin-A, in PB of BCC patients [36]. However, more recently, similar percentages of T cell subsets and NK cells were reported in the PB of BCC patients and controls of similar age [113]. T cell counts and proliferative responses decrease with advancing age, which may explain the apparent decrease in the T and NK cells described in the early studies [36,112]. These findings in the PB of BCC patients indicate that immune suppression, if it occurs at all, is a local rather than a systemic phenomenon. In the direct vicinity of BCC, a varying degree of inflammatory infiltrate, mainly consisting of T lymphocytes [114], is often present. The degree of this infiltrate does not correlate with the age and sex of the patient or with the size of the tumor. The presence of NK or B cells is very rare, but LC are often present around the tumor nests [36,114,115]. Such a T cell-mediated immune response is very common in most solid tumors [116]. Both $CD4^+$ and $CD8^+$ T cells are present in the peritumoral infiltrate in BCC with a CD4/CD8 ratio of ± 2 [115,117,118]. Most of the peritumoral inflammatory cells express HLA-DR [115] and other activation markers, such as CD25 (IL-2R), CD45RO and the transferrin receptor [117]. This indicates that at least a proportion of the T cells is activated. An effective T cell-mediated immune response requires a T cell-tumor cell interaction, for which expression of MHC class I molecules as well as costimulatory molecules, such as ICAM-1, on the tumor cells are necessary. In addition, it has been shown that MHC class II (HLA-DR) molecules play an imperative role in tumor immunology [110,119]. However, BCC cells do not express HLA-DR (MHC class II) and ICAM-1, and the expression of MHC class I antigen on tumor cells is inconsistent [120-123]. The lack or down-regulation of the expression of these molecules may explain the lack of an effective T cell-mediated immune response. Despite this lack of efficacy of the immune response, spontaneous regression of superficial and nodular types of BCC has been observed [118]. This active regression of BCC is characterized by disruption of the

normal palisaded arrangement of the cells at the periphery of the tumor nests accompanied by a lymphocytic infiltrate penetrating and surrounding the tumor nests [118]. A significantly increased number of CD3+ and CD4+ T cells and a significantly increased expression of CD25 were observed in regressing compared with non-regressing BCC.

1.5. Cancer invasive growth and metastasis

Invasion and metastasis formation in general

Invasive growth of a tumor is the penetration of tumor cells through the basement membrane (BM) into the underlying tissue, such as the ECM. This phenomenon is a prerequisite for the formation of metastasis. Tumor metastasis is the process by which tumor cells dislodge from the primary tumor, travel to a distant site via the circulatory system, and establish a secondary tumor [124]. Adhesion molecules (Table III) and proteolytic enzymes play a very important role in all these steps. The intercellular adhesion between epithelial cells may prevent the cell locomotion and thereby invasive growth into the ECM. In contrast, adhesion of the tumor cells to the ECM proteins may promote invasive growth. In the majority of carcinomas, the chance of metastasis is statistically related to the size of the tumor. Additional parameters are: tumor growth rate, morphology, kinetics, anatomic location [125] and tumor invasiveness [126]. The formation of blood vessels (angiogenesis) is necessary for the primary tumor to grow and for tumor cells to reach the bloodstream [124]. Angiogenesis is promoted by growth factors secreted by tumor cells, such as fibroblast growth factors, TGFs, angiogenin, VEGF and others [124,127].

Adhesion molecules

Various adhesion molecules play an essential role in invasion and metastasis of tumors. The major families of adhesion molecules are cadherins, CD44, integrins, the immunoglobulin superfamily and selectins.

Cadherins are calcium-dependent cell-cell adhesion molecules, which bind to each other via homophilic interactions. They play a very important role in tissue morphogenesis and homeostasis. Cadherin is now recognized as a superfamily, composed of an ever-increasing number of members [128]. The three most common subclasses of cadherins are E-cadherin which is expressed on adult epithelial cells, P-cadherin which is expressed by placenta and epithelia where it is primarily concentrated in the basal layers, and N-cadherin which is expressed by adult neural tissues and muscle [126]. E-cadherin is a 120 kD transmembrane protein, expressed by the large majority of mature epithelia. The gene

is located on chromosome 16q [128]. Linkage between E-cadherin and the actin filaments of the cytoskeleton is necessary to form strong cell-cell adhesion. This linkage is localized in adherens junctions and mediated by catenins (α,β, and γ), vinculin and actin [129]. The catenins are noncovalently linked to the intracellular domain of E-cadherin. Functional catenins are necessary for normally functioning E-cadherin. Deletion of the α-catenin gene leads to non-aggregating, non-adhesive cells, whereas phosphorylation of β-catenin leads to diffuse membrane expression of E-cadherin and increased cell dissociation, motility and invasion [128,10,131]. Several functional studies into the role of E-cadherin in the invasiveness of carcinoma cells in vitro have been reported [126,130]. Blocking of E-cadherin with specific antibodies was found to increase the invasiveness of the cells [130]. Transfection of E-cadherin cDNA into a highly malignant and invasive cell line with low or no expression of E-cadherin led to suppression of the tumorigenicity and invasion, respectively [132,33]. In vivo studies showed that in several carcinomas such as breast cancer, prostate cancer, SCC of the head and neck and gastric cancer, down-regulation of E-cadherin and/or α-catenin was associated with increased metastasis formation and with a poor prognosis [130,134-136]. Recently, LOH of the E-cadherin gene locus 16q22.1 was noted in carcinomas of the breast, prostate and liver [128,129,134]. On the basis of those and similar findings, E-cadherin can be regarded as an invasion and metastasis suppressive molecule.

CD44 designates a broadly distributed family of transmembrane glycoproteins that mediate cell-cell and cell-ECM adhesion, including lymphocyte homing, hemopoiesis, cell migration, and tumor metastasis [137,138]. It is a proteoglycan, which is known to bind to hyaluronate, collagen and fibronectin [138]. Alternative splicing results in at least 10 different mRNAs, which are translated into functionally diverse, but structurally similar proteins [139]. The most prevalent form of CD44, CD44*standard*, has a molecular mass of 90 kD and is predominantly expressed on leukocytes. Other isoforms (150-160 kD) are expressed by epithelial cells, including carcinoma cells and some hematopoietic cells [138]. In the skin, the different forms of CD44 are expressed in the basal and spinous cell layer of the epidermis and in the skin appendages [140]. The expression of some CD44 isoforms is highly associated with tumorigenicity and metastasis formation in several carcinomas [126,140]. In rats, a region of CD44 encoded by variant exon 6 (CD44V6) conferred metastatic potential to non-metastasizing pancreatic carcinoma cells and blocking with a CD44V6 specific antibody blocked the invasion of these cells [141].

Integrins are adhesion molecules, which consist of noncovalently associated α- and β-subunits. To date, 8 different β and 14 α subunits have been reported [142]. Certain α-subunits can combine with more than one β-subunit and most integrins can bind to more than one ligand [126]. Integrins function as cell-cell adhesion molecules, as receptors for

BM components such as collagen and laminin, and as receptors for ECM proteins such as fibronectin, fibrinogen, vitronectin and thrombospondin. The specificity of ligand binding of integrins is provided by the α-subunit [143] and they predominantly interact with their ligands at sites containing an Arg-Gly-Asp region (RGD) [142]. Integrins are the prime mediators of interactions between cells and their surrounding ECM [139]. In general, loss of integrins on tumor cells is associated with loss of normal contact with the BM, leading to increased invasive growth and metastasis. Individual members of the β_1 subfamily differ according to which one of the α-chains combines with the β-subunit. They are designated very late activation antigens (VLA), because they appear on the lymphocyte surface very late (2-4 weeks) after activation with antigens [144]. In the normal epidermis, the expression of VLA-2 ($\alpha_2\beta_1$), VLA-3 ($\alpha_3\beta_1$), both receptors for laminin and collagen, and VLA-5 ($\alpha_5\beta_1$), a receptor for fibronectin, are located at the lateral-apical membrane of the basal cell layer [142,143]. Expression of VLA-2 and VLA-3 was also noted on the keratinocytes of the sweat glands and the cells of the outer root sheath of the hair follicle [145]. In the normal skin, the laminin receptor, $\alpha_6\beta_4$, is restricted to the basal cell membrane of the basal keratinocytes and functions as a major linker of the epidermis with the BM and is strongly associated with hemidesmosomes [146]. The relation between the increased expression of $\alpha_6\beta_4$ and invasion of tumor cells was also shown in murine epidermal keratinocytes, intra-epithelial neoplasia of the cervix uteri in human and in SCC of the head and neck [142,146]. Integrin molecules may be involved in mediating the interaction between the neoplastic cells and the BM and the specialized stroma and may account for the peculiar growth and differentiation characteristics of tumor cells [145].

The immunoglobulin superfamily comprises a wide variety of molecules that share a common structure named the immunoglobulin homology unit. This structure consists of 70 to 110 amino acids, organized into 7 to 9 β-pleated sheets. Most members of this family are involved in cell-cell recognition and include molecules which participate in cellular immunity (MHC antigens, CD4, CD8 and the T cell receptor (TCR)), neural development (N-CAM), leukocyte trafficking (ICAM-1, ICAM-2, ICAM-3, vascular adhesion molecule (VCAM)-1) and signal transduction (colony-stimulating factor-1 receptor and platelet derived growth factor receptor). VCAM-1 is expressed on endothelial cells (EC) and acts as the counter-receptor of VLA-4. Up-regulation of VLA-4 expression in melanomas has been correlated with an increased metastatic potential [137,144,147,148]. In addition, in other tumor cell lines VLA-4/VCAM-1 interaction has been described in metastasis formation [144].

Increased expression of ICAM-1 on the tumor cells has been observed to correlate with increased tumor progression and increased risk of metastasis in melanoma, but the exact

mechanism of this phenomenon is unknown [149,150]. Normally, ICAM-1 plays a role in cell-mediated immunity to tumors via T cell-tumor cell interactions.
Selectins are adhesion molecules on the endothelial cells that normally play a role in the first contact of leukocytes (rolling) with EC. Certain tumors such as colon carcinomas express the sialylated $Lewis_X$ antigen, the ligand of E-selectin. Its expression was correlated with the metastasis formation in these tumors [126].

Table III: Adhesion molecules and their involvement in metastasis formation.

Adhesion molecule	Type of adhesion	Ligand
Cadherins	Cell-cell	Cadherins
CD44s	Cell-cell/Cell-ECM	Hyaluronate
		Collagen
		Fibronectin
Immunoglobulin superfamily	Cell-cell/EC-Cell	
VCAM-1		VLA-4
Integrins	Cell-cell/Cell-ECM	
VLA-1, VLA-2, VLA-3		Type IV collagen
		Laminin
VLA-4		VCAM-1
VLA-5		Fibronectin
$\alpha_6\beta_4$		Laminin
Selectins	EC-Cell	
E-selectin		Sialyl $Lewis_X$ antigen
		Sialyl $Lewis_A$ antigen
		CD15
P-selectin		Sulfatides

This table only shows those adhesion molecules, which have been reported to play a role in invasive growth and metastasis formation.

Extracellular matrix proteins and proteolytic enzymes

The ECM and its degradation play an important role in the invasion and metastasis of tumors. The ECM consists of the BM and the interstitial stroma, and contributes significantly to the tissue architecture. It has several biological functions and forms a mechanical barrier to infectious agents, host immune cells and invasion of tumor cells [127]. In the skin, it plays a role in attachment, growth, spreading and regulation of differentiation and gene expression in epithelial cells [143].

Major components of the BM are type IV collagen and laminin, whereas fibronectin and proteoglycans are more prominent in the interstitial stroma [127]. The interaction of tumor cells with the ECM occurs in multiple stages of the metastatic cascade. Tumor cells may have various effects on the ECM. These are 1) degradation of matrix components by the production of proteolytic enzymes; 2) stimulation of fibroblasts to accumulate matrix components (desmoplasia) and 3) synthesis of matrix components [125].

Several enzymes such as interstitial collagenases, stromelysins, gelatinases and type IV collagenases play a role in the degradation of ECM [127]. Interstitial collagenase degrades type I, II and III collagen and is produced by fibroblasts and some tumor cell lines. Stromelysin 1 and gelatinase B, a type IV collagenase, degrade BM components and interstitial collagens. Stromelysin 3 is produced in the stroma of various malignant tumors but no structural substrate in the ECM has yet been identified. Gelatinase B (MMP2) degrades the type IV collagenous stucture of the BM and other ECM components such as fibronectin and interstitial collagen [151]. All these enzymes are metal ion-dependent proteases and therefore are referred to as matrix metalloproteases (MMP). Plasminogen activator (PA) converts the pro-enzyme plasminogen into plasmin. Plasmin plays a role in the conversion of procollagenase and prostromelysin into collagenase and stromelysin, respectively [134,152]. Inhibition of collagenase and stromelysin is regulated by tissue inhibitors of MMP (TIMPs) [124,1152]. Up-regulation of MMPs and/or down-regulation of TIMPs may lead to proteolysis of the ECM proteins.

Adhesion molecules and proteolytic enzymes in BCC

Although BCC is a malignant tumor, it rarely metastasizes, but it shows infiltrative growth. As already mentioned, several adhesion molecules play a role in invasive growth and metastasis formation in tumors. Therefore, the expression of various adhesion molecules in BCC has been investigated in a number of studies.

E-cadherin expression was observed to be normal in superficial and nodular types of BCC, but was decreased in the infiltrative type in two studies [128,153]. In a different study also reduced expression of E-cadherin was observed in the tumor nests compared

with that in the normal epidermis in 28 out of 30 BCCs [154]. These findings of reduced E-cadherin expression, particularly in the infiltrative type [128,153], indicate that this may play a role in the growth pattern and the local aggressive behavior of some BCC.

CD44 expression was observed to be reduced in BCC and SCC compared with the normal epidermis [155-159]. Expression of CD44*standard* has been observed to be predominantly located in the central areas of the tumor nests [159,160]. In several carcinomas, increased expression of CD44 isoforms is highly correlated with the tumorigenicity [126,140]. In contrast, in BCC and SCC, the expression of CD44*standard* and its variant isoforms, $V_{3\text{-}6}$ and V_9, was shown to correlate with proliferation and an undifferentiated phenotype [158]. In these tumors, no correlation has been observed between CD44 expression and increased invasive growth or metastasis formation. Based on these observations, CD44 expression probably does not play a major role in these events in BCC and SCC.

Integrins: The expression of VLA-2 and VLA-3 is pericellular in BCC and SCC in contrast with their expression in the normal epidermis, where it is located on the basal side of the basal cells [142,146,161,162]. In BCC, VLA-3 was predominantly observed on the peripheral cells of the tumor nests [143,145]. However, in BCC with partial regression, no expression of VLA-2 and VLA-3 was observed in the foci of regression [145]. This indicates that the decreased expression of these molecules correlates with a disruption of the basal membrane surrounding the tumor nests.

VLA-5 ($\alpha_5\beta_1$) was not observed in nodular BCC [142,162] and focal expression in superficial BCC was observed in only one study [161]. However, strong expression of this integrin was found in SCC [142,146]. The integrin $\alpha_6\beta_4$ was absent in BCC, whereas in SCC, an invasive and metastasizing tumor, $\alpha_6\beta_4$ was strongly expressed [146,162]. This may indicate a positive correlation between VLA-5 and $\alpha_6\beta_4$ and invasive growth and metastasis formation. However, this has not been proven yet.

Expression of the *selectin* ligands sialyl $Lewis_X$, sulfatide-1, sialyl $Lewis_A$ and CD15 were investigated in both BCC and SCC. Sialyl $Lewis_X$ and CD15 were observed to be expressed in SCC but sulfatide-1 was expressed in only some of these tumors. Sialyl $Lewis_A$ was not expressed in SCC. In BCC, none of these ligands were expressed. However, in both BCC and SCC endothelial E-selectin was noted [163].

As mentioned previously, angiogenesis plays a role in tumor metastasis. VEGF is a dimeric glycoprotein that induces microvascular hyperpermeability, and is a selective EC mitogen, which is suspected to play an important role in the angiogenesis associated with malignant tumors [164]. An immunohistochemical study showed that the expression of VEGF in SCC was higher than that in BCC. The latter were always faintly positive [164].

It may be suggested that the low expression of VEGF in BCC perhaps contributes to the non-metastatic behavior of these tumors.

Investigations into the BM components in BCC showed variable results. Fetal antigen-2 (FA-2) is a 26 kD BM associated antigen, which is originally isolated from second trimester human amniotic fluid. In normal skin, it is located along the BM of the dermal-epidermal junction. In the dermis, it is located around blood vessels, hair follicles and eccrine glands [165]. FA-2 was identified as the aminopropeptide of the α_1 chain of type I procollagen [105]. In BCC, it was observed in the BM surrounding the tumor nests, except for areas with inflammatory infiltrate cells and degeneration of elastin [165]. In the same study, laminin and type IV collagen were found in the BM surrounding the tumor nests. However, Tanaka et al [166] showed that type IV collagen consists of 6 chains, α_1 (IV)-α_6 (IV), and that in normal skin, α_1 (IV), α_2(IV), α_5(IV) and α_6(IV) are co-localized in the BM.

In BCC, the expression of the different chains depended on the histopathological type of the tumor. In nodular BCC, the α_1(IV) and α_2(IV) chains were consistently present, but the staining for α_5(IV) and α_6(IV) chains was either very weak or totally absent. In superficial BCC, the α_1(IV) and α_2(IV) chains formed a thick continuous layer, whereas the α_5(IV) and α_6(IV) chains formed only a thin layer. In the morpheic type of BCC, staining for the α_1(IV) and α_2(IV) chains was predominantly continuous, but discontinuous in some areas, whereas the α_5(IV) and α_6(IV) chains were not observed at all. Recently, the presence of mRNA of different BM components was investigated in BCC [167]. The mRNA of bullous pemphigoid antigen (BPAG)-1 (230 kD) and BPAG-2 (180 kD), the α_6 and β_4 chain of the $\alpha_6\beta_4$ complex and the α_3 chain of laminin 5 were all observed to be decreased in BCC compared with the normal epidermis. These observations are largely consistent with the immunohistochemical findings.

All these alterations in the BM of BCC may lead to a structurally defective BM that facilitates the invasive growth of BCC. In addition, proteolytic enzymes such as gelatinase A [151], gelatinase B (type IV collagenase) [168,169], matrilysin [195], PA [171], stromelysin-3 [172], interstitial collagenase [173] and stromelysin-1 [173] were all detected in BCC using different techniques. The presence of mRNA of a protease inhibitor (TIMP-2) has also been reported in BCC [151]. These observations indicate that the combination of expression of certain adhesion molecules, ECM components, proteolytic enzymes and their inhibitors plays a pivotal role in the growth and non-metastatic behavior of BCC. However, the exact individual contribution of all these components remains to be elucidated.

1.6. Therapy of basal cell carcinoma

Physical intervention

Different strategies are employed to treat BCC. The treatment that is chosen depends on the location, size and histological type of the tumor. Cosmetic aspects, particularly when the tumor is located on the face, play also a very important role in the choice of therapy. Furthermore, recurrent BCCs often require treatment different from the treatment of primary tumors.

The most common treatment is surgical excision under local anesthesia, which is relatively rapid and usually definitive (Table IV). The entire resected specimen is available for pathological study to evaluate histological type and margins. MMS is undertaken to treat recurrent and aggressive types of BCC and for BCC located on difficult sites. Frederick E. Mohs developed this technique, which is very time consuming, in 1936 [174]. In MMS, the tumor is excised layer by layer and the entire depth of each layer is examined microscopically. Besides many advantages such as the low recurrent rate of the tumor, some serious disadvantages of this technique have been reported [175,176]. It has been described that sections obtained by MMS are difficult to interpret and may be misleading.

In cryosurgery the tumor is treated with liquid nitrogen. Small BCCs (< 2 cm in diameter) on certain sites, such as near the eye or on the auricle, are treated with cryosurgery. Although this technique is very rapid, a serious disadvantage is that pathological evaluation is not possible. Therefore, it is usually not used for treating infiltrative and recurrent BCCs [177]. However, recently the consequences of this therapy were investigated in 54 patients with 56 recurrent BCCs and favorable wound healing and excellent cosmetic results were observed. In only 2 patients recurrences were noted, 3 and 7 years after therapy [178].

Other treatment modalities are radiotherapy and curettage. In both instances pathological evaluation is impossible. Avril et al [179], in a randomized study in which BCC were either treated with surgery or radiotherapy, concluded that surgery was more effective. Photodynamic therapy using aminolevulinic acid and laser irradiation has been used in BCC [180,181].

Recently, it was shown that the effect of photodynamic therapy for BCC depends on both tumor thickness and the duration of the photosensitizer application [182]. In addition to these routine treatments, new experimental therapies such as with cis-platinum containing regimens [183] or with 5-fluorouracil [184] have also been reported.

The use of all these different therapies depends on the type of BCC and its location. However, surgical excision is still the most prominent therapy used.

Table IV: The most common therapies for BCC and the percentage of recurrences after 5 years [177,181].

Therapy	Percentages of recurrences 5 after:	
	Treatment of primary BCC	Treatment of recurrent BCC
Surgical excision	4.8-10.1	17.4
MMS	1.0	
Cryosurgery	7.5	5.6
Radiotherapy	7.4-8.7	9.8
Curettage	7.7-13.2	40.0
Photodynamic surgery	81	3.0

Immunotherapy

The most important aim of immunotherapy is to stimulate the immune system to destroy the tumor. In general, cytokine-based immunotherapy aims to boost the anti-tumor immune response mainly by stimulating T cell responses. Such therapies involve different approaches: 1) Direct administration of cytokines to the patients, either systemically or locally; 2) In vitro culture of immune cells from patients with cytokines, followed by re-administration of the lymphokine-activated killer (LAK) cells to the patients; 3) Transfection of cytokine genes into T cells in order to activate the local antitumor defense, and 4) Transfection of cytokine genes into tumor cells in order to stimulate and activate CTLs in vivo [185]. In addition, immunoregulatory cytokines such as IFN-γ and IL-2 have been used to overcome the effects of immunosuppressive cytokines [109].

In the last two decades, a number of clinical trials on intralesional treatment of BCC with IFN have been reported [186-198]. Such investigations were pursued in order to develop a treatment for BCCs with more satisfying cosmetic and functional results or for treatment of BCCs on body sites not easily accessible for surgery. Successful treatment of BCC with IFN-γ has been reported in several studies [187,189-194,197,198]. A total dose of 9 x 106 to 13.5 x 10^6 international units (IU) IFN-γ was administered intralesionally 2 or 3 times a week for 2 or 3 weeks to nodular or superficial BCCs. The percentage of complete tumor remission varied from 33 [189] to 100 [187]. The T cell-mediated immune response was enhanced after IFN-γ therapy as indicated by an increased number of peri- and intratumoral activated T cells [189]. However, the exact mechanism by which IFN-γ cures BCC is still unknown.

It was reported that IFN-γ has a higher tissue affinity than IFN-γ. This leads to higher levels of IFN-γ in the tumor and lower levels in the systemic circulation. This property might constitute a significant advantage for the treatment of BCC. To our knowledge, only one study reported intralesional treatment of BCC with IFN-γ [196]. In this study different doses and administration frequencies were used. A total dose of 1 x 10^6 IU and intralesional injections 3 times a week for 3 weeks led to complete remission in 12 of 14 patients. This was determined 12 weeks after initiation of the therapy.
Intralesional treatment of BCC with IFN-γ was reported in several studies [186,188]. Tank et al [186] observed only flattening of the tumor and an increase in peritumoral inflammatory infiltrate, but no reduction of the tumor size. However, Edwards et al [188], using different doses and frequencies of application, observed complete remission in 50% of treated patients.
From these studies it may be concluded that intralesional treatment with IFN-α or -β may be a useful therapy on selected cases such as BCCs located at difficult sites not easily accessible for surgery or in cases of multiple BCCs.

1.7. Aims of the study

The aims of the studies described in this thesis were,

1. *To clarify the cytokeratin expression pattern in BCC.* The results of studies published so far have been contradictory [95-101]. This may have been caused by the use of different immunohistochemical detection techniques and monoclonal antibodies (MAbs) with different epitope specificities. Therefore, in an attempt to resolve these discrepancies, we used a large panel of MAbs against Ck 7, 8, 18 and 19 in a comprehensive immunohistochemical study. In addition, an immunoelectron microscopic (IEM) study was undertaken to confirm the light microscopic (LM) findings on the expression of Ck 8 and its potential significance as a histopathological marker of BCC.

2. *To obtain additional insight into the observed lack of effective cell-mediated immunity in BCC in spite of the presence of varying degrees of peritumoral inflammatory infiltrate, predominantly consisting of T cells.* The aim was to investigate the possible reasons for the absence of or the rare presence of T cells within the tumor nests. These may be the absence or down-regulation of molecules on BCC cells, which play an essential role in the T cell-tumor cell interaction. These molecules are MHC class I & II antigens and the costimulatory molecules ICAM-1, CD40 and B7.1 (CD80). Furthermore, other factors

such as immunosuppressive cytokine IL-10 and soluble (s) ICAM-1, both of which may contribute towards inhibition of the cellular immune response, were investigated.

3. *To obtain a better insight into the non-metastatic behavior of BCC.* Despite the fact that BCC is a malignant tumor, metastases are very rare. In several tumors, such as carcinomas of the breast [136] and colon [131,199], the expression of certain adhesion molecules on the tumor cells is correlated with the metastatic behavior of these tumors. Loss of E-cadherin, α- and β-catenin expression and increased expression of $CD44V_6$ is correlated with an increased invasive growth and metastasis formation. Therefore, the expression of these adhesion molecules in BCC was examined. In addition, the observed down-regulation of these molecules on BCC cells compared with the overlying epidermis was investigated using IEM.

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Chapter 2

CYTOKERATIN EXPRESSION IN BASAL CELL CARCINOMA

2.1. *Expression of cytokeratin 8 and other low molecular weight cytokeratins in human basal cell carcinoma*

2.2. *Expression of cytokeratin 8 in basal cell carcinoma: A comparative immunolohistochemical and immunoelectron microscopic study*

CHAPTER 2.1

Expression of cytokeratin 8 and other low molecular weight cytokeratins in human basal cell carcinoma

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SUMMARY

The expression of low molecular weight Cks 7,8,18,19 and high molecular weight Ck 10 in 23 BCCs was investigated using a panel of 14 different commercially available MAbs with specific anti-Ck activity. Four of these MAbs were directed against Ck 8. The results showed that Ck 8 was detected in all 23 BCCs using MAb 4.1.18. Two of the MAbs showed inconsistent staining for Ck 8 and one of them did not show any staining at all. Cks 7 and 19 were detected inconsistently. Cks 18 and 10 were not detected in any of the 23 BCCs that were examined. The inconsistent observations on the expression of Ck 8 in BCCs in this study could have been due to different epitopes of the different cytokeratins that were detected by the different MAbs.
The results of this study lead to the recommendation that whenever possible a panel of different MAbs directed against the same Ck(s) should be used in order to minimize the risk of obtaining incorrect experimental results.

INTRODUCTION

BCC of the skin is one of the most common forms of skin cancer of which the incidence is still increasing worldwide. Its incidence in the United Kingdom was reported to be increased by 235% during the last 14 years [1]. BCC is a slowly growing malignant epithelial tumor, which fortunately rarely metastasizes. However, infiltrative growth leading to local destruction may occur if left untreated. There is no general consensus on the origin of BCC as yet. There are several speculations to its origin. It is thought to be derived from basal cells of the epidermis, pluripotent epidermal germ cells, surface epidermis, hair follicle epithelial cells, cells of the pilosebaceous tract, undifferentiated hair matrix [2], and the outer root sheath of the hair follicle [3]. Therefore, the availability of a specific histopathological marker for BCC would not only be helpful in elucidating its origin(s), but also for detecting residual tumor after treatment.
Cytoskeleton proteins of epidermal cells may harbor such a marker. The cytoskeleton of epithelial cells is characterized by the presence of Cks. Specific Ck patterns are characteristic of certain epithelial cell types and reviewed in detail elsewhere [4].
During the past decade, various investigations focusing on the expression of low molecular weight Ck 8 in BCCs have been reported. However, the results of these investigations were rather discrepant. Using biochemical techniques, Moll et al [5] detected small amounts of Ck 8 in some BCCs, and Asada et al [6] detected trace amounts of Ck 8 in 6 out of the 20 BCCs. The expression of Ck 8 in 15 out of the 19 BCCs was reported in only

a single immunohistochemical study [6], whereas the expression of Ck 8 was not observed in any of the other studies [3,7-12]. The discrepant results of these studies may be attributed to the differences in the MAbs and the techniques that were used. However, to date, it is still not clear whether Ck 8 (if expressed) is a suitable specific marker for BCCs. Therefore, the aim of this study was to resolve the reported discrepancies on the expression of Ck 8 in BCCs using four different commercially available specific anti-Ck 8 MAbs. The distribution of the other low molecular weight Cks 7,18,19 and the high molecular weight Ck 10 in BCCs was also examined.

MATERIALS AND METHODS

Twenty-three specimens of BCC were obtained by surgical excision from twenty-three patients, 14 males and 9 females, aged 33 to 95 years. A biopsy was taken and frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen. The diagnosis was confirmed histologically by examination of hematoxylin and eosin (H&E)-stained sections. Twenty-two of these BCCs were located on the head and neck region and one on the shoulder. The histological classification of the BCCs showed that thirteen were nodular type, five were adenoid type, one was superficial type, one was nodular/morphoeic type, one was nodular/adenoid type and two were superficial/nodular type.

Immunohistochemistry

Cryostat sections were stained using the Avidin-Biotin complex method as described elsewhere [13]. Briefly, 5 µm cryostat sections were placed on glass slides, air dried and fixed in acetone for 10 minutes at room temperature. The sections were then rinsed in phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween and were pre-incubated with 5% bovine serum albumin (BSA) in PBS for 30 minutes to minimize non-specific staining. Subsequently, the sections were incubated for 60 minutes with an optimal dilution of each of the specific MAbs shown in Table I. The sections were subsequently rinsed twice with PBS containing 0.05% Tween and then incubated for 30 minutes with 1:200 diluted goat-anti-mouse (GAM) biotin containing 3% normal goat serum (NGS) and 3% normal human serum (NHS).

The sections were again rinsed twice with PBS containing 0.05% Tween and incubated for 30 minutes with the Avidin-Biotin complex diluted 1:1:200 (DAKO-kits, DAKO A/S, Denmark) and then rinsed twice with PBS (without Tween). The peroxidase reaction was developed by incubating the sections with 3,3'-diaminobenzidine (DAB) at a concentration of 0.75 mg/ml and 0.25% hydrogen peroxide for 7 minutes in the dark.

The sections were rinsed with tap water and counter-stained for 15 seconds in Hematoxylin (Mayer) and rinsed with tap water again for 5 minutes. The sections were mounted in malinol after a step-wise dehydration in alcohol and xylol.
The controls consisted of normal human skin, which was processed using the same procedure. Normal human skin was obtained from patients undergoing breast reductions at the department of plastic surgery. Punch biopsies of the normal skin were frozen in liquid nitrogen and kept at –80°C till use. The staining of the eccrine sweat glands was used as a positive control. The negative controls comprised the omission of primary antibody and the omission of GAM immunoglobulin. Relevant isotype controls were also always included.

Table I: Dilution, specificity and source of the MAbs.

MAb	Dil	Subclass	Spec	Manufacturer
RCK105	1:160	IgG_1	Ck 7	Organon Technika, Boxtel, The Netherlands
CK7	1:160	IgG_1	Ck 7	Boehringer Mannheim, Mannheim, Germany
OVTL12/30	1:50	IgG_1	Ck 7	Sanbio, Uden, The Netherlands
M20	1:80	IgG_1	Ck 8	ICN Biomedicals, Cleveland, OH,USA
4.1.18	1:1000	IgG_1	Ck 8	Boehringer Mannheim, Mannheim, Germany
RPN1166	1:5	IgG_1	Ck 8	Amersham, Bucks, England, UK
35βH11	1:150	IgM	Ck 8	ITK diagnostics, Uithoorn, The Netherlands
RKSE60	1:40	IgG_1	Ck 10	ICN Biomedicals, Cleveland, OH, USA
CK2	1:20	IgG_1	Ck 18	Boehringer Mannheim, Mannheim, Germany
RGE53	1:50	IgG_1	Ck 18	ICN Biomedicals, Cleveland, OH, USA
RCK106	1:200	IgG_1	Ck 18	Organon Technika, Boxtel, The Netherlands
NCL5D3	1:50	IgG_{2a}	Ck 8.18.19	Sanbio, Uden, The Netherlands
Ks19.1	1:200	IgG_{2a}	Ck 19	ICN Biomedicals, Cleveland, OH, USA
RCK108	1:200	IgG_1	Ck 19	Organon Technika, Boxtel, The Netherlands

Dil = dilution; spec = specificity

RESULTS

The reactivity patterns of 9 out of the 14 MAbs with each of the 23 BCCs are shown in Table II. The reactivity patterns of the remaining 5 MAbs - RPN1166, RKSE60, CK2, RGE53 and RCK106 - are omitted from Table II because no staining of tumor cells was observed with these MAbs in any of the 23 BCCs that were examined.

Cytokeratin 8
MAb 4.1.18 stained more than 75% of the tumor cells in 21 (91%) of the 23 BCCs. More than 25% of the tumor cells were stained in the remaining 2 cases. MAb 35βH11 showed staining in more than 75% of the tumor cells in 17 (74%) of the 23 BCCs. More than 5% of the tumor cells were stained in 5 cases. An example of the staining pattern with these MAbs is shown in Fig. 1a and b. MAb M20 stained more than 5% of the tumor cells in only 6 (26%) of the 23 cases. MAb RPN1166, however, did not stain tumor cells in any of the 23 BCCs.

Cytokeratin 7
MAbs RCK105 and CK7 showed similar staining patterns. They both stained more than 25% of the tumor cells in 3 (13%) of the 23 BCCs. MAb OVTL12/30 stained more than 75% of the tumor cells in 11 (48%) cases. An example of the staining pattern is shown in Fig. 1c. In 1 case only 5% of the tumor cells were stained.

Cytokeratin 18
MAbs RGE53, CK2 and RCK106 did not stain any of the tumor cells in any of the 23 BCCs that were examined.

Cytokeratin 19
MAb Ks19.1 stained more than 5% of the tumor cells in 7 (30%) of the 23 BCCs and Ab RCK108 stained more than 5% of the tumor cells in 11 (48%) cases. All the tumors, which were stained with MAb Ks19.1 also, showed staining with MAb RCK108.

Cytokeratins 8, 18 and 19
MAb NCL5D3 stained more than 75% of the tumor cells in 18 (78%) of the 23 BCCs. More than 25% of the tumor cells were stained in 4 cases. The only tumor, which did not show any staining, was also negative with both MAbs Ks19.1 and RCK108. All the MAbs directed against Cks 7, 8, 18 and 19 stained the eccrine sweat glands, but did not stain the epidermis.

Cytokeratin 10

MAb RKSE60 did not stain tumor cells in any of the 23 BCCs that were examined. It did stain the suprabasal layers of the epidermis in 20 (87%) of the BCCs. An example of the staining pattern is shown in Fig. 1d. The 3 BCCs in which no staining was observed involved hyperplasia of the epidermis.

Table II: The percentage of stained tumor cells in each BCC with the different MAbs.

Sample No.	4.1.18	35βH11	M20	RCK 105	CK7	OVTL 12/30	NCL 5D3	Ks19.1	RCK 108
1.	>75	>75	0	0	0	0	>75	0	0
2.	>75	>75	0	50-75	50-75	>75	>75	>75	>75
3.	>75	25-50	0	0	0	>75	25-50	0	0
4.	>75	>75	0	0	0	>75	25-50	0	5-25
5.	>75	>75	>75	>75	>75	>75	>75	25-50	25-50
6.	>75	>75	>75	0	0	0	>75	0	0
7.	>75	>75	0	0	0	0	>75	0	5-25
8.	>75	>75	0	0	0	0	0	0	0
9.	50-75	>75	0	25-50	>75	>75	25-50	0	0
10.	25-50	25-50	0	0	0	0	25-50	0	0
11.	>75	>75	0	0	0	>75	>75	50-75	50-75
12.	>75	>75	0	0	0	0	>75	0	0
13.	>75	0	5-25	0	0	>75	>75	0	0
14.	>75	>75	5-25	0	0	>75	>75	5-25	50-75
15.	>75	>75	0	0	0	0	>75	0	0
16.	>75	>75	5-25	0	0	0	>75	0	0
17.	>75	>75	0	0	0	0	>75	0	0
18.	>75	5-25	0	0	0	>75	>75	0	>75
19.	>75	>75	0	0	0	5-25	>75	5-25	>75
20.	>75	25-50	0	0	0	0	>75	>75	>75
21.	>75	50-75	>75	0	0	>75	>75	>75	>75
22.	>75	>75	0	0	0	0	>75	0	50-75
23.	>75	>75	0	0	0	>75	>75	0	0

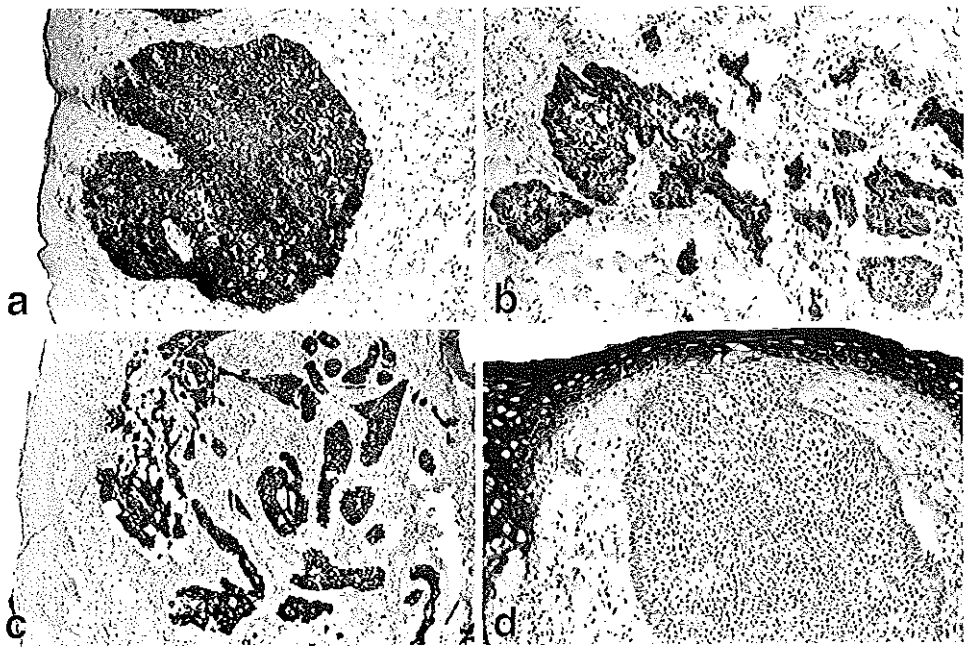

Figure 1: Cryostat sections (5μm) of nodular (a,c,d) and infiltrative (c) type of BCC, x 250. MAb 4.1.18 (a), MAb 35βH11 (b), and MAb OVTL12/30 (c) stained more than 75% of the tumor cells. MAb RKSE60 (d) stained the suprabasal layer of the epidermis, but the tumor cells remained negative.

DISCUSSION

The expression and the importance of Cks as potentially useful markers of various healthy tissues and diseases have been intensively investigated. Low molecular weight Cks were reported to be expressed in fetal epidermis, eccrine sweat glands of the adult skin [14], in epithelia of the liver, gall bladder, intestine and trachea [15] and in different adenocarcinomas [16]. Immunoreactivity to Cks was recently reported in non-epithelial cells of deep gastric ulcers [17]. Cks 8 and 18 were also reported to be present in smooth muscle cells [18].

The search for a specific histopathological marker for human BCC has received considerable attention. The availability of such a marker would be helpful in elucidating the origin(s) of this tumor in humans. Although the histological diagnosis of BCC in most

cases is straight forward, a suitable specific marker would be very useful for establishing correct diagnosis in complicated cases and for detecting residual tumor nests after surgical intervention. Therefore, various investigations focusing on the expression of Ck 8 were undertaken. The results of previous biochemical and light microscopy investigations were both inconclusive and discrepant. The investigations reported here were pursued in an attempt to clarify these inconsistencies. The expression of low molecular weight Cks 7, 8, 18 and 19 and high molecular weight Ck 10 was examined in 23 BCCs using a panel of 14 different commercially available MAbs.

The expression of Ck 8 was investigated using 4 different MAbs. The results showed that Ck 8 was expressed in all the 23 BCCs using MAb 4.1.18. Twenty-two (96%) out of the 23 BCCs were observed to stain for Ck 8 using MAb 35βH11, which was in contrast to the results reported by Murata et al [12] who did not observe any staining in any of the BCCs with this MAb. The difference may be attributed to the fact that those authors [12] had used paraffin-embedded sections and a higher dilution of the MAb. In this study, Ck 8 was not observed to be expressed in any of the 23 BCCs that were examined using MAb RPN1166. In a recent study, Asada et al [19] also failed to detect the expression of Ck 8 in any of the 20 BCCs with MAb RPN1166 using the alkaline phosphatase anti-alkaline phosphatase technique. In a previous study, Habets et al [8] also failed to detect the expression of Ck 8 in any of the 21 BCCs with MAb RPN1166. The same authors also failed to observe the expression of Ck 8 in any of the 5 BCCs with the same MAb in an IEM study [9]. In the present study, 6 (26%) out of the 23 BCCs were observed to be stained with MAb M20. The inconsistent staining with this MAb was also reported by Lavrijsen et al [6] who observed Ck 8 expression in 15 (79%) out of the 19 BCCs, whereas in a recent study others [10] reported that Ck 8 was not expressed in any of the 15 BCCs using the same MAb. At present, there is no satisfactory explanation for these inconsistencies except for the differences in the staining techniques that were used. The overall evaluation of the results on the expression of Ck 8 using the different MAbs in this study indicated that the discrepancies may be due to the different epitopes of Ck 8 that were detected by the different MAbs. Whether this is in fact the case still remains to be established.

Three different MAbs were used to examine the expression of Ck 7 in this study. Tumor cells of 3 (13%) out of the 23 BCCs were stained with MAbs RCK105 and CK7, indicating the expression of Ck 7. The intensity of staining varied and seemed to be more intense in the tumor cells situated in the deeper dermis. Similar varied staining within and between various tumor nests was also previously reported [8]. In the present study, 12 (52%) out of the 23 BCCs were observed to be stained with MAb OVTL12/30 indicating the presence of Ck 7 in BCC. This is in agreement with the results reported by Ramaekers

et al [16] who observed that relatively more cases were (partially) positive using MAb OVTL12/30 than using MAb RCK105. The different staining patterns observed could have been due to the different epitopes of Ck 7 that were detected by the different MAbs.
Ck 18 is usually co-expressed with Ck 8 [5,18,20]. Using MAbs RGE53, RCK106 and CK2, no expression of Ck 18 was observed in any of the 23 BCCs either in this study or in any of the previous studies [8-10]. Lavrijsen et al [6] used MAbs LE61 and M9 directed against Ck 18 and noted only a few positive cells in 1 (5%) out of the 19 BCCs. In the present study, MAb NCL5D3 which is directed against Cks 8, 18, and 19 showed staining in 22 (96%) out of the 23 BCCs. However, these results by no means establish the expression of Ck 18 because this MAb could have cross-reacted with other epitopes of Cks 8 or 19.
In this study, Ck 19 was also observed to be inconsistently expressed. Ck 19 was observed to be expressed in 7 (30%) and 11 (48%) out of the 23 BCCs using MAbs Ks19.1 and RCK108 respectively. All those BCCs, which were stained with MAb Ks19.1 also, showed staining with MAb RCK108. A possible explanation for the observation that more BCCs were observed to express Ck 19 using MAb RCK108 may well be that this MAb has additional activity against other epitopes of Ck 19. Habets et al [8] previously reported Ck 19 in 17 (81%) out of the 21 BCCs using MAb Ks19.1. Discrepant observations using the same MAb have also been previously reported. Using MAb BA17, Perkins et al [11] reported Ck 19 to be expressed in 6 (30%) out of the 20 BCCs, whereas Bartek et al [21] observed no expression of Ck 19 in 2 BCCs using the same MAb.
High molecular weight Ck 10 was not expressed in the tumor cells in any of the 23 BCCs using MAb RKSE60. This corroborates the results of previous investigations [8,9] in which the same MAb was used, but is in contrast to those reported in another study [6] in which only a few tumor cells were observed to be stained with the same MAb in 3 (16%) out of the 19 BCCs. However, no firm conclusion can be drawn from that study [6], but it suffices to say that the results of the present study are also corroborated by those reported in a recent study [3] in which Ck 10 was not detected in any of the BCCs using SDS-PAGE and immunoblotting techniques.
The results reported here allow the conclusion that the expression of Ck 8 was detected using MAbs 4.1.18 and 35βH11. However, it is advisable that, whenever possible and if available, a panel of different MAbs directed against the same Ck(s) should be used to minimize the risk of incorrect results. Thus, prior to assessing whether Ck 8 may serve as a specific immunohistological marker for BCC, its expression in BCC must be shown to be consistent.

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CHAPTER 2.2

Expression of cytokeratin 8 in basal cell carcinoma: A comparative immunohistochemical and immunoelectron microscopy study

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SUMMARY

The comparative study reported here was undertaken in order to resolve the discrepancies in the detection of Ck 8 reported in previous studies. The expression of Ck 8 was compared in 6 BCCs using immunohistochemical and IEM techniques and a panel of 4 different commercially available MAbs. The results of this comparative study demonstrated not only that the consistent expression of Ck 8 using one of the MAbs in immunohistochemistry was confirmed by immunoelectron microscopy, but that the inconsistent expression of Ck 8 observed using two other MAbs was also confirmed. One of the MAbs did not show any staining at all. The inability of this MAb to detect the expression of Ck 8 using either LM or IEM also indicated that this MAb may be directed against an epitope of Ck 8 that is not detectable in BCC in situ.

INTRODUCTION

BCC is one of the most common forms of skin cancer. Together with solar keratoses and squamous cell carcinoma, it poses a major health problem in fair-skinned individuals [1]. Its incidence in the United Kingdom was reported to be highly increased during the last 14 years [2]. BCC is a slowly growing malignant epithelial tumor of unclear origin. It is thought to be derived from the epidermis, hair follicle epithelial cells, cells of the pilosebaceous tract, undifferentiated hair matrix [3] and the outer root sheath of the hair follicle [4]. Although this tumor rarely metastasizes, there is a potential risk for recurrences after surgical intervention. Residual tumor cells after therapy are a likely cause of recurrence. Therefore, a specific histopathological marker for BCC would not only be valuable for detecting these residual tumor cells, but also for elucidating its origin(s). The possibility of Ck 8 being such a histopathologic marker for BCC was investigated using different techniques in various studies [4-11].

Recently, we investigated the expression of Ck 8 in 23 BCCs using a panel of 4 different commercially available MAbs directed against Ck 8 [12]. The results showed that the expression of Ck 8 was inconsistent and ranged from being present in all the examined BCCs (100%) to none (0%) of the BCCs depending on the MAb. The discrepancies in the results may have been due to the different epitopes of Ck 8 that were detected by the 4 MAbs or due to the limitations of the immunohistochemical technique that was used.

In previous studies, Haftek et al [13] and Habets et al [14] used IEM to investigate the expression of different Cks in normal epidermis and BCCs respectively. These authors

concluded that IEM is a sensitive and suitable confirmatory technique for immunohistochemistry.
The aim of this study was two-fold. Firstly, to compare and to confirm the expression of Ck 8 in BCCs that was observed in our previous study [12] and secondly, to try and resolve the discrepancies that were observed in that study. Therefore, in the study reported here, we compared the detection of Ck 8 in 6 BCCs using both LM and IEM and the same panel of MAbs. The results were similar using both the techniques simultaneously.

MATERIALS AND METHODS

Six specimens of BCC were obtained by surgical excision from 6 patients, 3 males and 3 females, aged 57 to 76 years. Two biopsies were taken from each BCC. The first was frozen in liquid nitrogen-cooled isopentane, stored in liquid nitrogen, and was processed further for immunohistochemistry.
The second biopsy was processed using the method described previously [15] with some modifications. It was fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 hours and then transferred into 4% paraformaldehyde in 2.3 M sucrose in 0.1 M Sorrensen buffer and was processed further for IEM. The tissue blocks were transferred into 2.3 M sucrose 30 minutes prior to sectioning. The diagnosis was confirmed histologically by examination of H&E-stained sections.

Immunohistochemistry

Cryostat sections were stained using the Avidin-Biotin complex method as described previously [16]. Briefly, 5 μm cryostat sections were placed on glass slides, air dried, and fixed in acetone for 10 minutes at room temperature. The sections were then rinsed in PBS containing 0.05% Tween, and were pre-incubated with 5% BSA in PBS for 30 minutes to minimize non-specific staining. Subsequently, the sections were incubated for 60 minutes with an optimal dilution of each of the specific MAbs shown in Table I. The sections were subsequently rinsed twice with PBS containing 0.05% Tween and then incubated for 30 minutes with 1:200 diluted GAM-biotin containing 3% NGS and 3% NHS.
The sections were again rinsed twice with PBS containing 0.05% Tween and incubated for 30 minutes with the Avidin-Biotin complex, diluted 1:1:200 (DAKO-kits, DAKO A/S, Denmark), and then rinsed twice with PBS (without Tween). The peroxidase reaction was developed by incubating the sections with DAB at a concentration of 0.75 mg/ml and 0.25% hydrogen peroxide for 7 minutes in the dark. The sections were rinsed with tap water and counter-stained for 1 minute in hematoxylin (Mayer, Fluka A.G., Buchs,

Germany) and rinsed with tap water again for 5 minutes. The sections were mounted in malinol after a step-wise dehydration in alcohol and xylol.
The controls consisted of normal human skin, which was processed using the same procedure. Normal human skin was obtained from patients undergoing breast reductions at the department of plastic & reconstructive surgery. Punch biopsies of the normal skin were frozen in liquid nitrogen and kept at -80°C till use. The staining of the eccrine sweat glands was used as a positive control. The negative controls comprised the omission of primary antibody and the omission of GAM immunoglobulin. Relevant isotype controls were also always included.

Immunoelectron microscopy
Ulltrathin cryosections (± 50 nm) were cut with glass knives using a Reichert FCS Ultracut Ultramicrotome (Leica, Rijswijk, The Netherlands) at -90°C to -100°C and transferred to carbon coated formvar filmed 200 mesh copper grids in 2 mm wire loop with a droplet of 2.3 M sucrose. The grids were then transferred to a droplet of 2% (W/V) gelatine solution in 0.1 M Sorrensen buffer for 5 minutes to remove the sucrose. Then they were rinsed thrice for 2 minutes in a droplet of PBS containing 0.15% (W/V) glycine and 0.5% (W/V) BSA. The sections were then incubated for 2 hours with undiluted specific MAbs shown in Table I. Subsequently they were rinsed thrice for 2 minutes in PBS containing 0.15% glycine and 0.5% BSA. The sections were then incubated for 30 minutes with 1:25 diluted 10 nm gold-labeled GAM IgG/IgM or GAM IgG (Aurion, Wageningen, The Netherlands), depending on the isotype of the primary antibody used. Then they were rinsed 4 times for 5 minutes in PBS containing 0.15% glycine and 0.5% BSA, followed by 3 rinses of 1 minute each with distilled water.
Sections were counter-stained with uranyl-oxalic acid (pH 8.0) for 10 minutes, then rinsed for 1 second in distilled water and then they were transferred into uranyl acetate (pH 4.0) for another 10 minutes. The grids were subsequently floated on a 1.5% methyl cellulose solution for a few seconds and scooped up on wire loops of diameter 3-3.5 mm. Access fluid was then removed using a filter paper until gold to blue interference colors developed on the grids. The sections were air dried and examined in a Zeiss 902 electron microscope operated at 80 KV.
Control sections were treated with 10 nm GAM-gold probe only. Background labeling was negligible. Areas of sections showing dark gold granule deposits were regarded as positive for Ck 8. The background labeling was very low and was similar to that in the controls in which the omission of the primary MAb resulted in the presence of sporadic or no gold granules.

Table I: The dilution and source of the MAbs against Ck 8 used for LM.

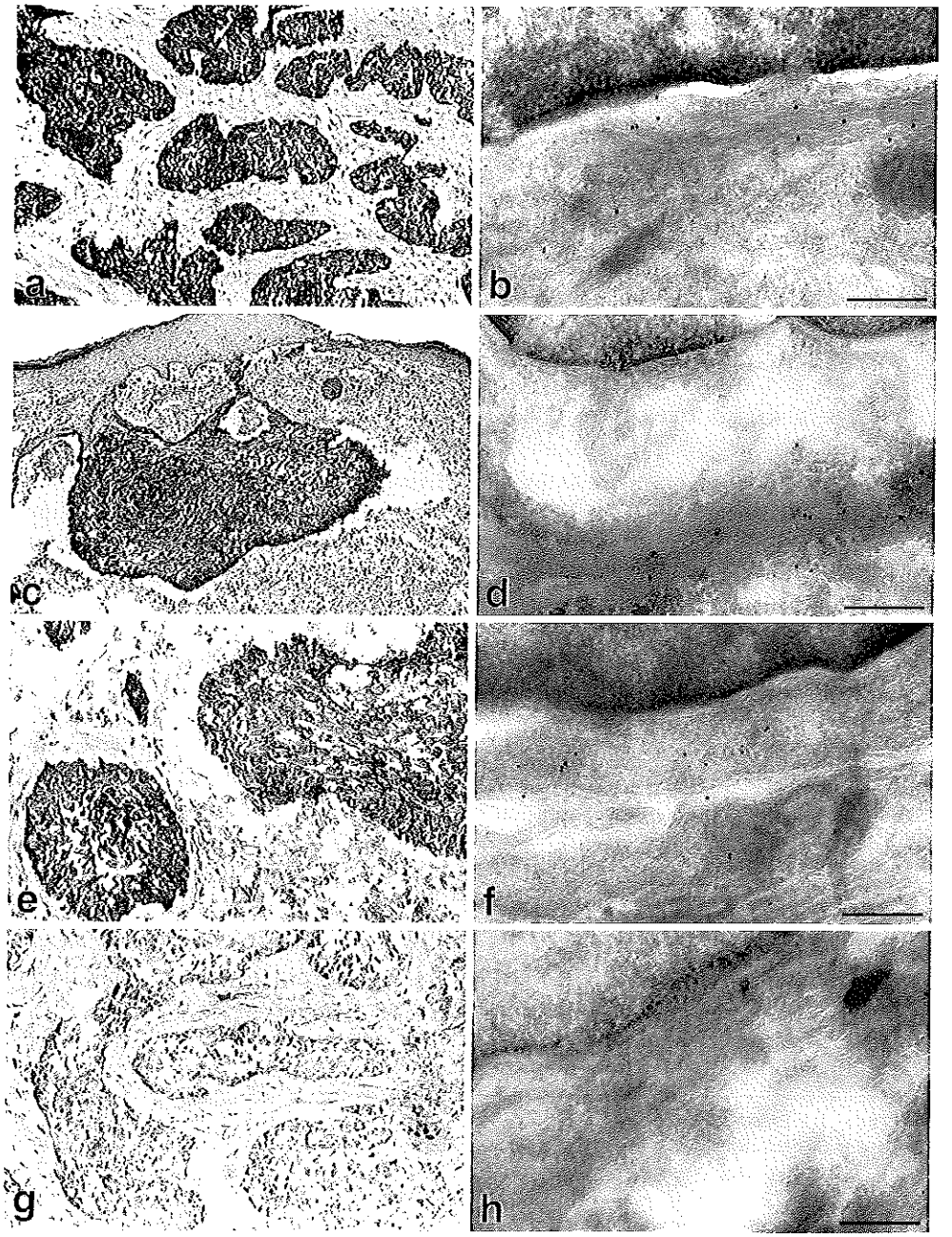
MAb	Dilution	Subclass	Manufacturer
RPN1166	1:5	IgG_1	Amersham, Bucks, England, U.K.
M20	1:80	IgG_1	ICN Biomedicals, Cleveland, OH, U.S.A.
35βH11	1:150	IgM	DAKO (ITK diagnostics), Uithoorn, The Netherlands
4.1.18	1:1000	IgG_1	Boehringer Mannheim, Mannheim, Germany

For IEM the same MAbs were used undiluted.

RESULTS

The characteristics of the six patients, the individual location and the type of the BCC are shown in Table II. A comparison of the reactivity pattern of the 4 MAbs directed against Ck 8 in the six BCCs that were examined is also presented in Table II. It can be seen that identical results were obtained with the same MAb using either of the techniques.
Two representative examples of the staining patterns of MAb 4.1.18 of the same tumor using immunohistochemistry and IEM are shown in Fig. 1a & b and 1c & d respectively. In Fig. 1a & c, it can be seen that all the tumor cells were stained indicating the expression of Ck 8. Fig. 1b & d represent the respective electron micrographs of the same tumors as those in Fig. 1a & c and they clearly show that the distribution of the gold granules was specifically restricted to the intermediate filaments. No gold granules were observed in the nucleus. After immunohistochemistry, MAb 35βH11 was observed to stain all the tumor cells in 5 (83%) out of the 6 BCCs that were examined. In the remaining tumor, about 75% of the tumor cells were stained. A representative example of the staining pattern with MAb 35βH11 indicating the expression of Ck 8 is shown in Fig. 1e. After IEM, the distribution of the gold granules was specifically observed to be restricted to the intermediate filaments in 4 (67%) out of the 6 BCCs indicating that Ck 8 was detected. Fig. 1f represents an electron micrograph of the same tumor as shown in Fig. 1e, which clearly shows that the distribution of the gold granules was specifically restricted to the intermediate filaments. In one of the BCCs there was inconsistent distribution of the gold granules, whereas no gold granules were observed in the remaining BCC.

Figure 1: Cryostat (a,c,e,g; x 250) and ultracryotomy (b,d,f,h; bar = 0.4 µm) sections of BCC stained with anti-Ck 8 MAbs 4.1.18 (a-d), 35βH11 (e,f) and RPN1166 (g,h). *(see page 68)*


a
b
c
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e
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g
h

Inconsistent staining at LM level and inconsistent distribution of gold granules at IEM level were observed with MAb M20 in only 1 (17%) of the six BCCs indicating an inconsistent expression of Ck 8, whereas no expression of Ck 8 was observed in the remaining 5 BCCs.
The expression of Ck 8 was not detected in any of the BCCs that were examined with MAb RPN 1166 using both the techniques. It can be seen in Fig. 1g that none of the tumor cells were stained by immunohistochemistry. Fig. 1h represents an electron micrograph of the same tumor and shows the absence of gold granules on the intermediate filaments.

Table II: Patients characteristics and a comparison of the results of the staining with the MAbs against Ck 8 in LM and IEM.

Patient	Age	Sex	Location	4.1.18		35βH11		M20	
				LM	EM	LM	EM	LM	EM
1.	57	F	Neck	++	++	++	++	±	±
2.	70	F	Right ear	++	++	++	++	--	--
3.	76	F	Right cheek	++	++	++	++	--	--
4.	66	M	Fore head	++	++	++	--	--	--
5.	66	M	Left temple	++	++	±75%	±	--	--
6.	71	M	Left eye corner	++	++	++	++	--	--

All BCC were of the nodular type. MAb RPN1166 did not show any staining at all in both LM and EM. + = all the tumor cells were stained (LM) and gold granules were present on the intermediate filaments (EM); - = no staining (LM) and no gold granules were observed (EM); ± = inconsistent staining (LM) and incidental gold granules were observed (EM).

DISCUSSION

Although the histological diagnosis of BCC in most cases is straight forward, a suitable specific histopathological marker would be very useful for establishing the correct diagnosis in complicated cases, for detecting residual tumor nests after surgical intervention and in elucidating the origin(s) of this tumor. BCC an epithelial tumor and the cytoskeleton of the epithelial cells is characterized by the presence of Cks.
The search for a specific histopathological marker for human BCC has received considerable attention during the last decade. A number of investigations focusing on the

expression of Ck 8 were undertaken with different and confusing results [4-11,17]. In a recent study [12], the expression of Ck 8 was investigated in 23 BCCs using a panel of 4 different commercially available MAbs. The results showed that the expression of Ck 8 in BCC was inconsistent and dependent on the MAb that was used.

In a previous study, Habets et al [14] used IEM to investigate the expression of Cks 7,8,18 and 19 in BCCs in an attempt to confirm the results of their earlier reported LM study [8]. The results of their IEM study confirmed the absence of Ck 8 in BCC, but in contrast to the results of their LM study, Ck 19 was detected in the normal human epidermis. Their explanation was that it was possible that Ck 19 was expressed in low amounts in normal epidermis and that it was not detected in immunohistochemistry because of the limitations in the resolution of that technique. Their observation, together with the discrepancies in the expression of Ck 8 that were observed in our recent study [12], prompted us to undertake the investigation into the expression of Ck 8 in the same BCC using LM and IEM techniques simultaneously.

To our knowledge, this is the first study of its kind in which the expression of Ck 8 has been investigated using both these techniques. The results of the immunohistochemistry showed once again that the expression of Ck 8 varied depending on the MAb that was used and ranged from expression in all six cases to none in all six cases, corroborating our recent observations [12]. The results of IEM were essentially the same and also confirmed those that were observed in LM using the same MAb. A comparison of the results obtained using the two techniques also confirmed the inconsistent expression of Ck 8 in BCCs.

At present, there is no satisfactory explanation for the observed inconsistencies except that it may be due to different epitopes of Ck 8 being detected with the four MAbs that were used. Whether this is in fact the case still remains to be established. Evidence for the presence of more than one epitope of Ck 19 in human tumors and cultured cells was reported in an earlier study by Bartek et al [18].

A firm conclusion on the inconsistent expression of Ck 8 in BCCs cannot be drawn from the results reported here, as similar comparative studies on the expression of Cks are not available at present. A definite answer on the possible value of Ck 8 as a suitable and specific histopathological marker for BCC must therefore await additional investigations in which different approaches are compared.

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Chapter 3

IMMUNOLOGY OF BASAL CELL CARCINOMA

3.1. *Expression of interferon-gamma receptors and interferon-gamma-induced up-regulation of intercellular adhesion molecule-1 in basal cell carcinoma: Decreased expression of IFN-γR and shedding of ICAM-1 as a means to esape immune surveillance.*

3.2. *Interferon-γ-induced ICAM-1 and CD40 expression, complete lack of HLA-DR and CD80 (B7.1) and inconsistent HLA-ABC expression in basal cell carcinoma: A possible role for IL-10?*

CHAPTER 3.1

Expression of Interferon-gamma receptors and Interferon-gamma-induced upregulation of Intercellular Adhesion Molecule-1 in basal cell carcinoma:

Decreased expression of IFN-γR and shedding of ICAM-1 as a means to escape immune surveillance.

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SUMMARY

The peritumoral inflammatory infiltrate in BCC of the skin consists mainly of T lymphocytes, which hardly invade the tumor nests. The absence of ICAM-1 on BCC cells may explain the lack of tumor-infiltrating cells and the lack of an active cell-mediated immune response in this tumor. In this study, the induction of ICAM-1 was investigated in BCC biopsies using recombinant human (rHu)IFN-γ. The expression of IFN-γ receptors (IFN-γR) in the biopsies was also investigated. The results showed that BCC cells expressed ICAM-1 after incubation with rHuIFN-γ, but to a lesser degree than normal epidermal cells. The levels of shed ICAM-1 were significantly increased in the culture supernatants of tumor biopsies as compared with those from normal skin biopsies, after culturing in the presence of rHuIFN-γ. The expression of IFN-γR on the tumor cells and the shedding of ICAM-1 into the peritumoral stroma may be a plausible mechanism by which the tumor cells are protected from an active cell-mediated immune response.

INTRODUCTION

BCC is the most common skin cancer in humans. The variable peritumoral inflammatory infiltrate in BCC mainly consists of T lymphocytes [1-3], but T cell invasion of tumor nests or T cells in close apposition with the tumor cells, are rarely observed. Together with molecules such as MHC class I & II antigens and B7, ICAM-1 is an essential molecule in facilitating cytotoxicity towards tumor cells [4-6]. IFN-γ and TNF-α have been shown to up-regulate ICAM-1 expression on keratinocytes in vivo [7-9] and in vitro [10,11], both membrane-bound and sICAM-1 [12]. Previous studies showed that BCC cells either did not express, or only occasionally expressed ICAM-1 [8,13-16]. The lack of ICAM-1 expression in BCC may explain the absence of an active cell-mediated immune response against this tumor. This absence of ICAM-1 may be caused by low local levels of cytokines, such as IFN-γ, as suggested by Kikuchi et al [14], or by a decreased expression of IFN-γR on the tumor cells. It is also possible that BCC cells have an intrinsic inability to express ICAM-1, or that they shed it in a soluble form into the peritumoral stroma.

The aim of this study was to examine whether ICAM-1 expression could be induced on BCC cells by rHuIFN-γ. The expression of IFN-γR on BCC and the shedding of ICAM-1 in the culture supernatants of the short-term biopsy cultures were also investigated.

MATERIALS AND METHODS

Short-term culture

At least 3 biopsies of 3 mm diameter each, from 20 BCCs, were obtained from 18 patients, 11 males and 7 females, aged 43 to 97 years. Normal (control) skin was obtained from 15 of these patients. In 13 patients, biopsies were taken from the area adjacent to the BCC and from 7 of these patients biopsies were also taken from the inner side of the upper arm (non-sun-exposed). From 2 patients, skin was obtained from only non-sun-exposed areas. Normal control skin was obtained from healthy patients undergoing breast reductions, in order to perform time- and dose-finding experiments. One of the biopsies of BCC and normal human skin was immediately snap-frozen in liquid nitrogen and stored at -80°C (t=0). The other biopsies were placed in a transwell-culture system (Costar, Badhoevedorp, The Netherlands) in a 2 mm perforation, such that the dermis was immersed in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Paisley, Scotland) containing 1% NHS in the presence or absence of rHuIFN-γ (Boehringer Ingelheim, Alkmaar, The Netherlands), whereas the epidermis remained exposed to the air. The biopsies were cultured for 24, 48 and 72 h at 37°C in an atmosphere of 5% CO_2 and 98% humidity. After incubation, they were carefully removed, frozen in liquid nitrogen, and stored at -80°C. The culture medium was centrifuged for 10 min at 1,500 g. The supernatant was transferred to a polypropylene tube and stored at -80°C. Pilot experiments showed that the viability and morphology of the biopsies cultured as described above remained unchanged at least for 72 h.

Immunostaining

For single-staining purposes, cryostat sections (6 μm) were cut and fixed for 10 min at room temperature in acetone containing 0.1% hydrogen peroxide. The sections were stained using the immuno-peroxidase streptavidin-biotin complex method, as described previously [17]. The following MAbs were used: MAb LB-2, IgG_{2b} isotype, (Becton Dickinson, Erembodegem-Aalst, Belgium), against ICAM-1; MAb 4.1.18 (Boehringer Mannheim, Mannheim, Germany) against Ck 8, which has been shown to stain BCC cells [17], and a MAb against IFN-γR (Genzyme Diagnostics, Sanbio, Uden, The Netherlands), both IgG_1 isotype. The intensity of the staining was scored by two independent investigators and graded as shown in Tables I and II.

A double-staining technique was used to confirm that ICAM-1 was expressed on BCC cells. Cryostat sections (6 μm) were acetone-fixed, preincubated with 5% BSA, incubated with MAb LB-2, and stained using the streptavidin-biotin complex method with β-

galactosidase as the substrate (0.72% ferry/ferro cyanide, 2.5% 5-bromo-4-chloro-3-indolyl β-galactoside, 0.11% magnesium chloride in PBS (pH 7.4)). After the sections had been rinsed with PBS (20 min), they were incubated with MAb 4.1.18 and stained with the alkaline phosphatase/anti-alkaline phosphatase method (Dako) using new fuchsine (Chroma Gesellschaft) as the chromogen. The sections were embedded in Kaiser's glycerol-gelatin (Merck, Darmstadt, Germany). They were rinsed twice with PBS between antibody incubations. Negative controls comprised the omission of primary MAbs. Relevant isotype controls were also always included.

Enzyme-linked Immunosorbent Assay (ELISA) for soluble ICAM-1
The level of sICAM-1 was determined in the supernatants of the biopsy culture media collected after culturing a biopsy in the presence or absence of rHuIFN-γ, using a specific and sensitive (<0.35 ng/ml) human sICAM-1 ELISA (R&D systems, Abingdon, U.K.). IMDM containing either 1% NHS or 1% NHS and 600 U/ml rHuIFN-γ was also stored and assayed in parallel as blank controls.

Statistical analysis
The results were analyzed using the Wilcoxon test for paired observations (WT) or the Mann-Whitney U test (MWUT). The level of significance, representing two-tailed testing, and the number of samples (n) are given in the Results.

RESULTS

Kinetics and induction of ICAM-1 expression in normal human skin
In normal uncultured human skin (t=0), ICAM-1 expression was limited to dermal EC. The up-regulation of ICAM-1 expression on epidermal cells of normal human skin, obtained from patients undergoing breast reductions, using various doses of rHuIFN-γ, was examined in order to establish optimal culturing conditions and dose of rHuIFN-γ (Fig. 1). In the presence of rHuIFN-γ, a dose-dependent up-regulation of ICAM-1 on the epidermal keratinocytes was observed, with no obvious difference between 24 h and 48 h of incubation. Optimum ICAM-1 expression in the epidermis was reached at doses of 500-700 U/ml rHuIFN-γ. A dose of 600 U/ml rHuIFN-γ was therefore chosen to investigate the induction of ICAM-1 in BCC biopsies.
Normal skin biopsies from non-sun-exposed areas from patients with BCC, and biopsies from skin adjacent to BCC were also investigated. A highly significant increase in ICAM-1 expression on epidermal cells of normal skin biopsies adjacent to BCC ($P \leq 0.01$, n=13,

WT) and on epidermal cells of normal skin biopsies from non-sun-exposed sites from patients with BCC (P≤0.01, n=9, WT) was seen after culturing in the presence of rHuIFN-γ, compared with that on epidermal cells of the biopsies cultured in the absence of rHuIFN-γ.

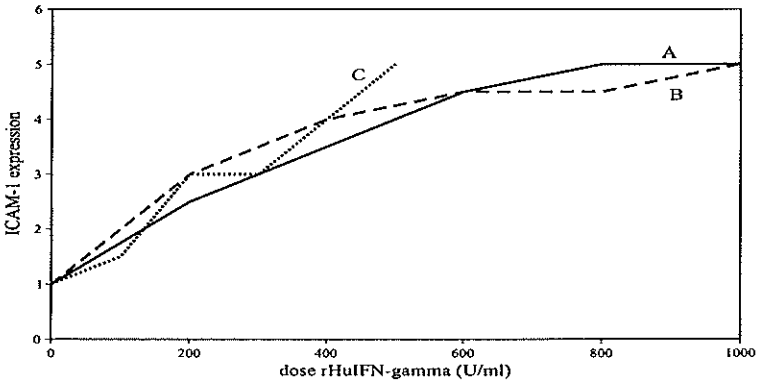

Figure 1: ICAM-1 expression in the epidermis of normal skin biopsies obtained from patients undergoing breast reductions. The staining of ICAM-1 was scored from 0 to 6 as follows: 0 = no staining; 1 = part of the stratum basale; 2 = stratum basale and the lower layers of the stratum spinosum; 4 = stratum basale and stratum spinosum; 5 = all viable layers of the epidermis; 6 = entire epidermis. The results of three separate experiments are shown. In experiment A, the biopsies were cultured for 24 h, and in experiments B and C for 48 h.

Induction of ICAM-1 expression in BCC biopsies

At baseline (t=0), the tumor cells and the overlying epidermis were almost entirely negative for ICAM-1. Infiltrating cells, endothelial cells, fibroblasts and the peritumoral stroma expressed ICAM-1. ICAM-1 expression in BCC biopsies after culturing for 24 h in presence or absence of 600 U/ml rHuIFN-γ is shown in Table I. A highly significant increase in ICAM-1 expression in the presence of rHuIFN-γ in the overlying epidermis (P≤0.01, n=14, WT) was observed, compared with that in BCC biopsies cultured in the absence of rHuIFN-γ. In 17 of the 20 BCC biopsies (85%) cultured in the presence or rHuIFN-γ, the ICAM-1 expression on the tumor cells, mainly at the periphery of the tumor nests, was significantly increased (P≤0.05, n=20, WT) compared with tumor cells in the biopsies cultured in the absence of rHuIFN-γ. Fig. 2a and 2b show double-stained sections of BCC after culturing for 24 h in the absence and presence of rHuIFN-γ, respectively. It can be seen that ICAM-1 is indeed also expressed on the tumor cells, which are stained for Ck 8 [17]. ICAM-1 staining in the peritumoral stroma was also increased when the biopsies were cultured in the presence of rHuIFN-γ.

Table I: The expression of ICAM-1 in the epidermis overlying the tumor nests and on the tumor cells after 24 h of culture

Sample No.	In the absence of RHuIFN-γ		In the presence of rHuIFN-γ		rHuIFN-γ-induced increase	
	Epidermis	Tumor	Epidermis	Tumor	Epidermis	Tumor
1.	1	1	3	2	2	1
2.	1	1	3	3	2	2
3.	NP	1	0	3	-	2
4.	0	1	4	2	4	1
5.	1	1	3	2	2	1
6.	2	0	4	2	2	2
7.	0	0	2	2	2	2
8.	2	0	3	1	1	1
9.	1	0	0	2	-1	2
10.	0	1	2	2	2	1
11.	1	0	1	1	0	1
12.	1	1	1	3	0	2
13.	0	0	3	2	3	2
14.	0	1	1	2	1	1
15.	1	0	2	1	1	1
16.	1	3	NP	0	-	-3
17.	0	1	1	0	1	-1
18.	1	3	4	1	3	-2
19.	NP	2	NP	3	-	1
20.	NP	0	NP	3	-	3
Mean	0.765	0.850	2.176	1.850	1.563	1.000
SE	0.161	0.209	0.324	0.209	0.316	0.324

Expression of ICAM-1 was scored from 0 to 4 as follows: 0 = no staining; 1 = 0-25% stained; 2 = 25-50% stained; 3 = 50-75% stained and 4 = >75% stained. NP = not present in the biopsy; - = could not be calculated; SE = standard error.

Table II: The expression of the IFN-γR in the epidermis overlying the tumor nests and on the tumor cells

Sample No.	Epidermis overlying the tumor nests		Tumor
	Basal	Suprabasal	
1.	3	3	1
2.	3	3	2
3.	3	3	1
4.	0	0	1
5.	3	1	1
6.	2	1	1
7.	2	2	1
8.	3	2	2
9.	2	2	1
10	2	2	1
11.	3	3	0
12.	3	3	2
13.	2	2	2
14.	2	2	1
15.	3	2	1
16.	3	2	2
17.	3	2	1
18.	3	2	1
19.	3	2	1
20.	3	2	1
Mean	2.55	2.05	1.20
SE	0.169	0.169	0.117

Expression of the IFN-γR was scored from 0 to 3 as follows: 0 = no expression; 1 = weak expression; 2 = moderate expression; 3 = strong expression. SE = Standard error.

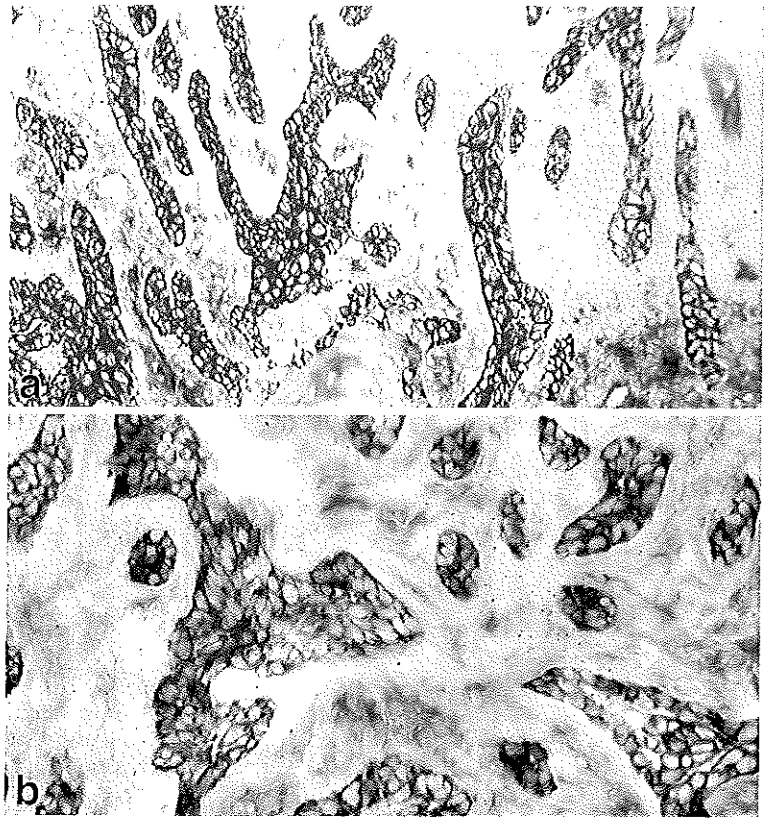

Figure 2.

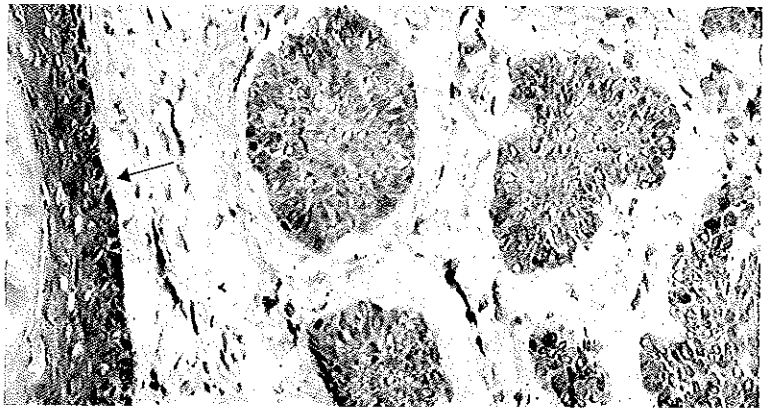

Figure 3.

Expression of IFN-γ receptors in BCC

In Table II, it can be seen that IFN-γRs were expressed both in the overlying epidermis and on the tumor cells in 19 out of the 20 BCC biopsies examined by immunostaining. However, their expression on the tumor cells was significantly lower than that in the basal layer ($P \leq 0.001$, n=19, WT) and the suprabasal layers ($P \leq 0.01$, n=15, WT) of the overlying epidermis. There were no differences in the expression of IFN-γR between biopsies from the same tumor cultured in the presence or absence of rHuIFN-γ. A representative example of the staining pattern is shown in Fig. 3. IFN-γR expression in the overlying epidermis was comparable to that observed in normal skin biopsies.

Shedding of ICAM-1 in the culture supernatants

The levels of sICAM-1 determined by ELISA in the culture supernatants of biopsies of normal skin and BCC are summarized in Fig. 4. The level of sICAM-1 in the supernatant of BCC biopsies cultured for 24 h in the presence of 600 U/ml rHuIFN-γ was significantly increased compared with supernatants from biopsies cultured in the absence of rHuIFN-γ ($P \leq 0.01$, n=19, WT). The degree of peritumoral inflammatory infiltrate was graded as mild, moderate, or heavy in H&E-stained cryosections of all 20 tumors (results not shown). As shown in Fig. 5, no correlation was observed between rHuIFN-γ-induced increase in sICAM-1 and the degree of peritumoral inflammatory infiltrate (Spearman's rank correlation test, correlation coefficient = 0.353, n=20).

The rHuIFN-γ-induced increase in sICAM-1 in the culture supernatants of BCC biopsies (n_2) was significantly higher than that induced in normal skin biopsies from non-sun-exposed sites from patients with BCC (n_1) ($P=0.046$, $n_1=9$, $n_2=20$, MWUT). The culture supernatants of biopsies of normal skin adjacent to BCC cultured in the presence of rHuIFN-γ also showed a significant increase in the level of sICAM-1 ($P=0.05$, n=6, WT) compared with those of biopsies cultured in the absence of rHuIFN-γ. However, the increase in the level of sICAM-1 was lower than that in the supernatants of BCC biopsies.

Figure 2: Double-stained cryostat section of a BCC cultured for 24 h without (a) and with (b) 600 U/ml rHuIFN-γ. The tumor cells are stained red (MAb 4.1.18, alkaline phosphatase) and the blue staining shows ICAM-1 expression (MAb LB-2, β-galactosidase)

Figure 3: Cryostat section of a BCC and the overlying epidermis stained for IFN-γR (MAb IFN-γR). The staining of the epidermis (arrow) was more intense than that on the tumor cells.

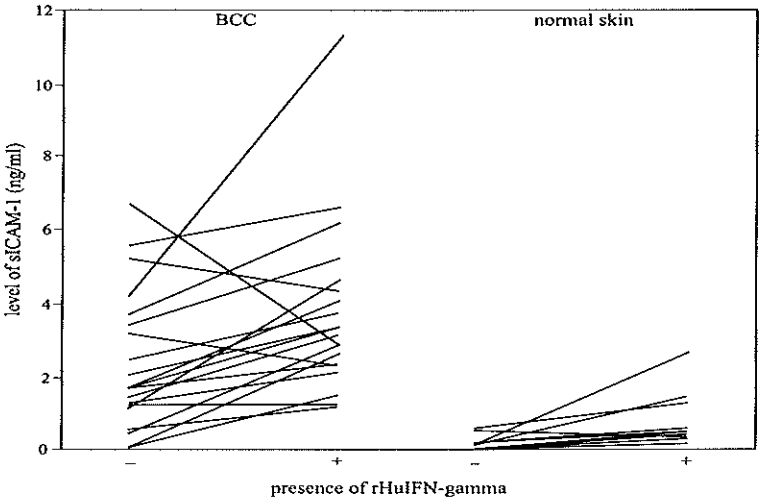

Figure 4: Level of sICAM-1 in the culture media of BCC biopsies and biopsies of normal skin after culturing for 24 h in the absence or presence of rHuIFN-γ

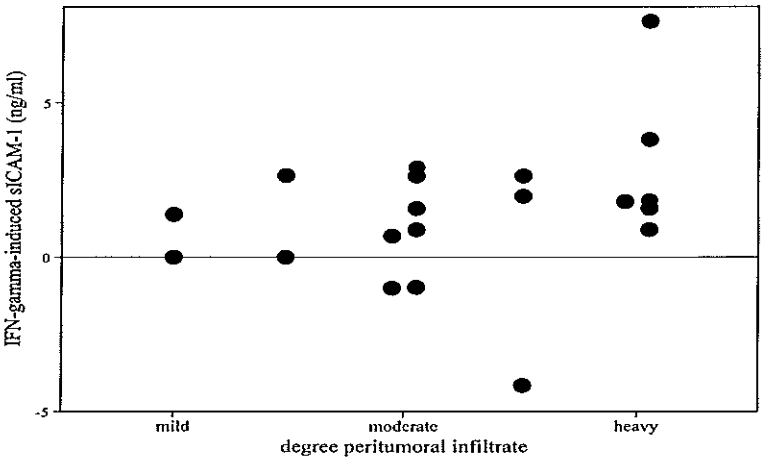

Figure 5: Correlation between the degree of infiltration and the IFN-γ-induced sICAM-1 after culturing in the presence of 600 U/ml rHuIFN-γ. There is no correlation between these parameters (Spearman's correlation coefficient = 0.3530, n = 20)

DISCUSSION

The investigations described in this report were undertaken in order to explore the reasons for the lack of ICAM-1 expression in BCC in vivo. The results showed that BCC cells did not have an intrinsic inability to express ICAM-1. ICAM-1 was observed to be expressed on the tumor cells of BCC biopsies that were cultured in the presence of rHuIFN-γ using a novel culture system. However, ICAM-1 expression was significantly higher both in the epidermis overlying the tumor nests and in the epidermis of normal skin, compared with the tumor cells. In addition, it was also confirmed that ICAM-1 was indeed expressed on BCC tumor cells by double staining.

There are two possible explanations for the difference in the up-regulation of ICAM-1 expression between the tumor cells of BCC, the overlying epidermis, and the epidermis of normal skin. First, there may be decreased expression of IFN-γR on the tumor cells compared with the overlying epidermis and the epidermis of normal skin. At present, to our knowledge, no studies are available on the expression of IFN-γR in BCC. However, the presence of this receptor in normal and psoriatic skin has been reported in two different studies [18,19]. The results of our study showed that the expression of IFN-γR on the tumor cells of BCC was significantly decreased compared with the overlying epidermis and the epidermis of normal skin. The low expression of IFN-γR, or a differential defective intracellular signaling of the receptor complex on BCC cells, may account for reduced responsiveness of these cells to rHuIFN-γ. This, in turn, may be an explanation for the observed reduced up-regulation of ICAM-1 by rHuIFN-γ on BCC cells compared with the overlying epidermis.

Secondly, ICAM-1 may be shed as a soluble form, sICAM-1, into the peritumoral stroma. The results showed that sICAM-1 was present in supernatants collected after culturing BCC biopsies in the absence of rHuIFN-γ. This may represent the level of sICAM-1 that was already present in the peritumoral stroma at the time that the biopsies were taken and probably had leaked out spontaneously into the media during culturing. The presence of sICAM-1 in the peritumoral stroma in situ at baseline (t=0) was also confirmed by immunohistochemical staining (Figure 2). The level of sICAM-1 in the supernatants was significantly increased after culturing BCC biopsies in the presence of rHuIFN-γ. It may well be that the sICAM-1 is derived from keratinocytes in the overlying epidermis [12] and/or peritumoral inflammatory infiltrating cells. However, the rHuIFN-γ-induced increase in sICAM-1 in the supernatants collected after culturing BCC biopsies was significantly higher than after culturing non-sun-exposed healthy skin biopsies from patients with BCC under the same conditions (Figure 4). The level of sICAM-1 in the supernatants after culturing these normal skin biopsies in the absence of rHuIFN-γ was

negligible. The degree of inflammatory infiltrate was greater in the normal skin adjacent to BCC than in non-sun-exposed normal skin. These infiltrating cells could have been the source of the observed higher rHuIFN-γ-induced level of sICAM-1 in the supernatants of these biopsies after culturing, compared with that of non-sun-exposed skin biopsies. However, there was no correlation between the increase in sICAM-1 and the degree of peritumoral inflammatory infiltrate in BCC biopsies, indicating that it is less likely that the observed increase in sICAM-1 was derived from infiltrating cells. Budnik et al [20] reported that shedding of ICAM-1 occurred by proteolytic cleavage of membrane-bound ICAM-1. Proteolytic enzymes such as gelatinase A and stromelysin were reported to be present in the peritumoral stroma in BCC [21-23]. Although, at present, there is no direct evidence, it is plausible that these or other enzymes may cleave membrane-bound ICAM-1 on the tumor cells of BCC to form sICAM-1.

It can be stated that sICAM-1 probably plays an important role in the lack of effective cell-mediated immunity in BCC by trapping T cells in the stroma [24,25], whereby interaction with tumor cells is prevented [26]. A specific local cocktail of cytokines may also be responsible for the lack of ICAM-1 expression on the tumor cells in situ. To date, there have been no reports on the presence of IFN-γ in BCC. However, it was reported that T cells in the peritumoral infiltrate in BCC expressed high levels of IFN-γ and IL-2 mRNA, whereas tumor cells predominantly expressed IL-4 and IL-10 mRNA. High levels of IL-10 in culture supernatants of BCC cell lines [27] and TGF-β [28,29] in BCC have been reported. These may have an immunosuppressive effect on the T cells in the peritumoral inflammatory infiltrate.

In conclusion, the decreased expression of IFN-γR and the increased levels of sICAM-1 observed in the present study may be means by which local T cell-mediated immune response is evaded in BCC. Other factors might include a low local amount of IFN-γ and the presence of immunosuppressive cytokines.

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CHAPTER 3.2

Interferon-γ-induced ICAM-1 and CD40 expression, complete lack of HLA-DR and CD80 (B7.1) and inconsistent HLA-ABC expression in basal cell carcinoma:

A possible role for Interleukin-10?

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SUMMARY

BCC of the skin shows varying degrees of peritumoral inflammatory infiltrate, mainly consisting of T cells, but lack an effective T cell-mediated immune response. This may be caused by the absence of key molecules known to play an important role in T cell-tumor cell interactions, like the MHC class I & II antigens and the costimulatory molecules ICAM-1, CD40 and CD80 (B7.1). All these molecules have been shown to be induced or up-regulated by IFN-γ on epithelial cells, while their expression can be down-regulated by IL-10. In this study, we investigated the induction and up-regulation of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 in BCC and normal skin from BCC patients in a culture system using rHuIFN-γ. The levels of IL-10 were determined in the supernatants after culture. The results showed that only ICAM-1 expression was significantly up-regulated on BCC cells. However, in the normal epidermis of BCC patients and in the epidermis overlying the tumor nests significant up-regulation of ICAM-1, CD40 and CD80 and a slight up-regulation of HLA-DR were observed. No changes in HLA-ABC expression were observed in either normal skin or BCC. High levels of IL-10 were present in the supernatants of BCC biopsies cultured for 48 hours without rHuIFN-γ. Culturing with rHuIFN-γ resulted in a significant inhibition of the IL-10 levels in the supernatants of both BCC and normal skin biopsies from BCC patients. It may be concluded that it is highly likely that the presence of IL-10 in BCC is directly or indirectly responsible for the complete lack of expression of HLA-DR, ICAM-1, CD40 and CD80 and the inconsistent expression of HLA-ABC on BCC cells in situ and may be a way for escaping immune surveillance.

INTRODUCTION

BCC is the most common form of skin cancer. The tumor nests of BCC are surrounded by a varying degree of inflammatory infiltrate mainly consisting of T cells [1,2]. These T cells are only sporadically seen in close apposition to or within the tumor nests indicating the lack of an effective cell-mediated immune response against this tumor. MHC class I & II antigens and costimulatory molecules such as ICAM-1, CD40 and CD80 (B7.1) have all been shown to play an important role in T cell activation and proliferation [3-6]. Normally, MHC class I antigens are expressed on all somatic cells; MHC class II antigen expression is restricted to B cells, activated T cells and antigen-presenting cells (APC); ICAM-1 is expressed on EC; CD40 is expressed on APC and its expression has also been observed on activated keratinocytes [7-9]; CD80 is not expressed on keratinocytes. In

inflammatory skin diseases, like psoriasis and lichen planus, expression of HLA-DR and ICAM-1 [10] and up-regulation of CD40 expression on keratinocytes [7] has been observed. This is induced by pro-inflammatory cytokines such as IFN-γ and TNF-α. The effect of IFN-γ on the expression of HLA-DR and ICAM-1 on keratinocytes [10,11] and of CD80 on gastric carcinoma cells [12] has been confirmed in vitro.
In BCC, absence or down-regulation of MHC class I & II antigens [13-16], ICAM-1 [17,18] and CD40 [7,9] has been reported, but to our knowledge, the expression of CD80 in BCC has not yet been investigated. In addition to this lack or low expression of these molecules on BCC cells, the production of IL-10 may inhibit an effective cell-mediated immune response. IL-10 has been shown to down-regulate the expression of ICAM-1 and HLA-DR on melanoma cells [19] and of CD80 on gastric carcinoma cells [12]. Therefore, the presence of IL-10 may be the cause of the discrete level of expression of these molecules in BCC. At present, studies on the cytokine pattern in BCC are scarce. Kim et al [20] reported the presence of IL-10 mRNA in the BCC cells and high levels of IL-10 in the culture media of BCC cell lines. In contrast, Yamamura et al [21] did not detect IL-10 mRNA in BCC cells, but instead in the peritumoral T cells.
The aim of our study was to investigate the expression of adhesion molecules and the function of cytokines in BCC using a novel biopsy-culture system [22]. In this system, BCC and normal skin biopsies from BCC patients were cultured with rHuIFN-γ to induce the expression of MHC class I (HLA-ABC) & II (HLA-DR) antigens, ICAM-1, CD40 and CD80 on the tumor cells. In addition, the levels of IL-10 in the supernatants after culturing the BCC and normal skin biopsies from BCC patients for 48 h with or without rHuIFN-γ were measured.

MATERIALS AND METHODS

Short-term culture

At least 3 biopsies of 3 mm each, from 20 BCCs, were obtained from 20 patients, 9 males and 11 females, aged 39 to 94 years. Normal skin was obtained from the inner side of the upper arm from 10 of these patients. Time-kinetic and dose-finding experiments were performed using normal (control) skin obtained from healthy patients undergoing breast reductions. One of the biopsies of BCC and normal skin was immediately snap-frozen in liquid nitrogen and stored at -80°C (t=0). The other biopsies were placed in a transwell-culture system (Costar, Badhoevedorp, The Netherlands) and were cultured in IMDM (Gibco, Paisley, U.K.) containing 1% NHS as described previously [22]. After culturing the biopsies for 48 h with or without rHuIFN-γ (Boehringer Ingelheim, Alkmaar, The

Netherlands), they were carefully removed, snap-frozen in liquid nitrogen and stored at -80°C. The culture medium was centrifuged for 10 minutes at 1,500 g. The supernatant was transferred to a polypropylene tube and stored at -80°C.

Immunostaining

For single-staining purposes, cryostat sections (6 μm) were cut and stained using the immunoperoxidase streptavidin-biotin complex method as described previously [23]. The following MAbs were used: LB-2 (ICAM-1), IgG_{2b} isotype (Becton Dickinson, Erembodegem-Aalst, Belgium); HLA-DR, IgG_{2a} isotype (Becton Dickinson, Erembodegem-Aalst, Belgium); NCL-HLA-ABC (HLA-ABC), IgG_{2a} isotype (Novocastra Laboratories Ltd, Newcastle, U.K.); MAb 104 (CD80), IgG_1 isotype (Immunotech, Marseille, France) and MAb 5D12 (CD40), IgG_1 isotype (PanGenetics BV, Amsterdam, The Netherlands). The intensity of the staining was scored by two independent investigators and graded from 0 to 5 as follows: 0 = no staining; 1 = up to 20% stained; 2 = 21-40% stained; 3 = 41-60% stained; 4 = 61-80% stained and 5 = more than 80% stained.

Double staining was used to determine the cell type expressing the particular molecule. Cryostat sections (6 μm) were cut and stained using the double-staining technique with β-galactosidase and new fuchsine as substrates as described previously [22]. The above MAbs were used in addition to CD3, IgG_1 isotype (Becton Dickinson, Erembodegem-Aalst, Belgium) and MAb 4.1.18, IgG_1 isotype (Boehringer Mannheim, Mannheim, Germany) against Ck 8, which was reported to stain BCC cells [23].

Enzyme-linked immunosorbent assay (ELISA) for IL-10

The levels of IL-10 were determined in the supernatants of the biopsy culture media collected after culturing a biopsy for 48 h in IMDM containing 1% NHS with or without rHuIFN-γ, using a specific and sensitive (<1.5 pg/ml) human IL-10 ELISA (R&D systems, ITK Diagnostics, Uithoorn, The Netherlands). IMDM containing 1% NHS was assayed in parallel as blank control.

Statistical analysis

The results were analyzed using the WT, the MWUT or the Spearman's rank correlation test (SRCT). The level of significance, representing two-tailed testing, and the number of samples (n) are mentioned in the Results. A p value of lower than 0.05 was considered to be statistically significant.

RESULTS

Induction and expression of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 in normal skin biopsies

Time-kinetic and dose-finding experiments were performed with normal (control) skin, obtained from healthy patients undergoing breast reduction, to determine optimal conditions for the investigations into the induction and up-regulation of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 on BCC cells. Immunohistochemistry showed that in normal uncultured skin, HLA-ABC was expressed in all epidermal layers; HLA-DR was expressed on infiltrating cells in the dermis and on langerhans cells in the epidermis; ICAM-1 expression was restricted to dermal EC; CD40 expression was observed only in the basal layer of the epidermis and CD80 expression was not detected at all.

HLA-DR expression on keratinocytes was up-regulated after 48 h of culture with rHuIFN-γ (Fig. 1) and therefore, the expression of all molecules was examined after 48 h of culture. The optimal dose for the up-regulation of all the relevant molecules was 900 U/ml rHuIFN-γ as is shown in Fig. 2. Therefore, this dose was used in all further experiments in this study.

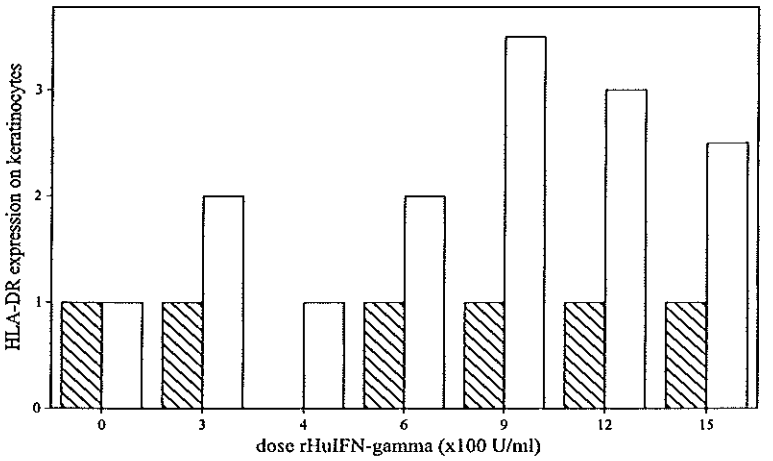

Figure 1: HLA-DR expression in the epidermis of normal (control) skin biopsies cultured for 24 h and 48 h with different doses of rHuIFN-γ varying from 0 to 1500 U/ml. The expression was scored as described in Materials and Methods

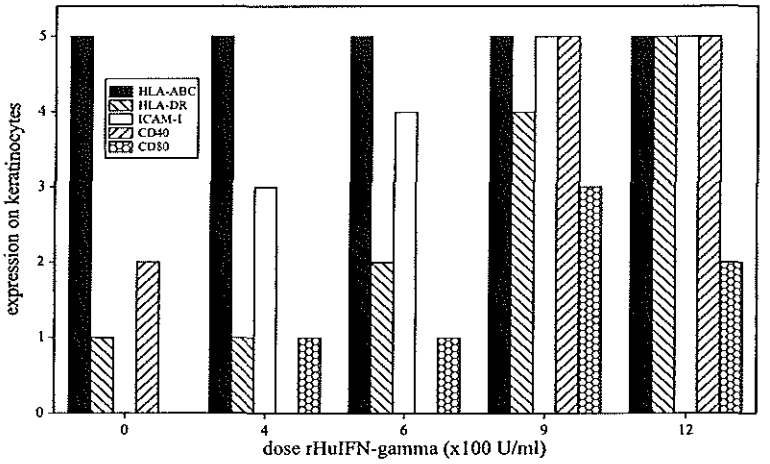

Figure 2: The expression of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 in the epidermis of normal (control) skin biopsies cultured for 48 h with different doses of rHuIFN-γ varying from 0 to 1200 U/ml. The expression was scored as described in the Materials and Methods.

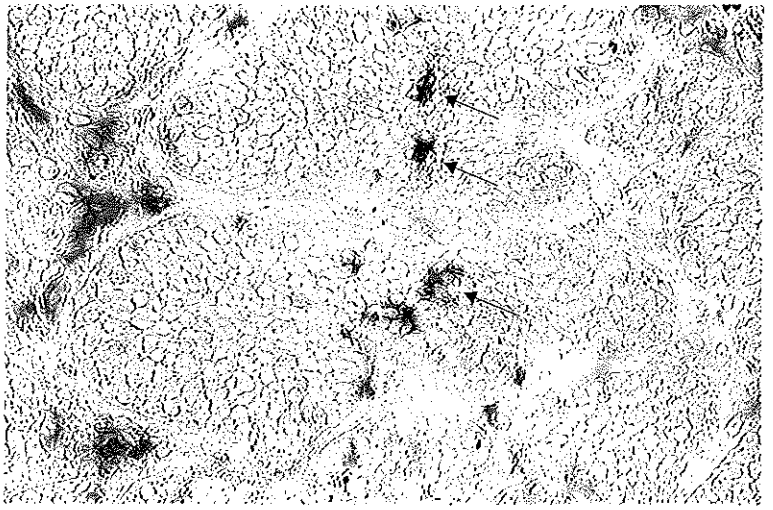

Figure 3: Double-stained cryosection of BCC cultured for 48 h with 900 U/ml rHuIFN-γ stained for HA-DR (β-galactosidase, blue) and CD3 (alkaline phosphatase, red). The stained cells within the tumor nests express both molecules, indicating the incidental presence of T lymphocytes within the tumor nests.

At baseline (t=0), the expression of all the relevant molecules in the epidermis of normal skin from BCC patients before culture was comparable with that in the normal (control) skin described above. Table I shows the increase in the expression of the examined molecules after 48 h of culture with 900 U/ml rHuIFN-γ compared with the expression after culturing without rHuIFN-γ. The increase in both ICAM-1 and CD40 expression in the epidermis was highly significant ($p \leq 0.01$, n=9, WT). Only a trend was seen in the increase in the expression of HLA-ABC ($p \leq 0.2$, n=4, WT), HLA-DR (p=0.2, n=4, WT) and CD80 (p=0.1, n=5, WT). However, induction of CD80 expression on the cells of inflammatory infiltrate in the dermis was observed.

Table I: Relative increase in the expression of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 in the epidermis of normal skin biopsies from BCC patients cultured for 48 h with 900 U/ml rHuIFN-γ compared with that of unstimulated biopsies.

Sample No.	HLA-ABC	HLA-DR	ICAM-1	CD40	CD80
1.	-2	-	4	3	0
2.	0	1	4	3	1
3.	0	0	1	3	0
4.	0	-	3	3	1
5.	-	-	4	3	1
6.	ND	2	5	5	1
7.	1	0	5	1	-
8.	2	2	4	2	1
9.	1	2	4	2	0
Mean	0.29	1.17	3.78	2.78	0.63
SE	0.44	0.37	0.38	0.34	0.17

The expression of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 was scored from 0 to 5 as described in the Materials and Methods. - = could not be calculated; SE = standard error; ND = not done.

Induction and up-regulation of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 in BCC biopsies

At baseline (t=0), strong staining for HLA-ABC, HLA-DR, ICAM-1 and CD40 was observed on peritumoral infiltrate cells. Keratinocytes in all layers of the epidermis overlying the tumor nests showed strong membrane staining for HLA-ABC in 11 (84.6%) of the 13 cases. In the other 2 cases the epidermis was only partly stained. HLA-DR expression in the overlying epidermis was restricted to the LC. In 10 (62.5%) of the 16 cases weak staining for ICAM-1 was observed on the keratinocytes in the basal layer of the overlying epidermis. Strong staining for CD40 was observed on the keratinocytes in the basal layer of the overlying epidermis in all cases. No staining at all was observed for CD80. Highly variable HLA-ABC expression on the BCC cells was observed in 7 (46.7%) of the 15 cases. This expression varied between 10% to 100% of the tumor cells. In all cases, the staining of the tumor cells was more diffuse and of a lower intensity than that observed in the overlying epidermis. In several BCC biopsies, a few cells stained for HLA-DR, CD40 and ICAM-1 were observed within the tumor nests. However, double staining showed that these stained cells were all CD3-positive, indicating that they were activated T cells. Thus, at baseline, there was no expression of HLA-DR, ICAM-1, CD40 and CD80 on BCC cells.

Table II shows the increase in the expression of the different molecules in BCC biopsies stimulated for 48 h with 900 U/ml rHuIFN-γ in relation to unstimulated biopsies. There was no difference in HLA-ABC expression in the epidermis overlying the tumor nests after culturing the BCC biopsies with or without rHuIFN-γ. HLA-DR expression in the epidermis overlying the tumor nests was not significantly up-regulated and an increased expression of HLA-DR on cells in the tumor nests was observed in only one case. Double staining showed that these were incidental tumor infiltrating T cells (Fig. 3). The increase in ICAM-1 expression in both the epidermis overlying the tumor nests (n=10) and on the BCC cells (n=16) was highly significant ($p \leq 0.01$, WT). CD40 expression in the epidermis overlying the tumor nests was significantly up-regulated ($p \leq 0.05$, n=8, WT). However, its expression was only slightly increased on the tumor cells in 4 (26.7%) of the 15 cases. An example of positively stained BCC cells for CD40 after incubation with rHuIFN-γ is shown in Fig. 4. The CD80 expression in the epidermis overlying the tumor nests was significantly increased ($p=0.01$, n=8, WT) after culturing with rHuIFN-γ, but BCC cells remained negative. An increase in CD80 expressing peritumoral infiltrate cells was noted. The increase in the expression of all molecules in the epidermis overlying the tumor nests was not significantly different from that in the epidermis of the normal skin biopsies from the BCC patients (MWT).

Table II: Relative increase in the expression of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 in BCC biopsies cultured for 48 hours with 900 U/ml rHuIFN-γ compared with that in unstimulated BCC biopsies.

Sample	HLA-ABC		HLA-DR		ICAM-1		CD40		CD80	
No.	Epi.	Tum.	Epi.	Tum.	Epi.	Tum.	Epi.	Tum.	Epi.	Tum.
1.	-	0	-	-1	-	2	-	0	-	0
2.	0	0	1	0	2	1	1	2	1	0
3.	0	-	2	0	4	2	4	-	2	-
4.	-	-5	-	0	-	1	-	3	-	1
5.	-	1	-	0	4	1	3	1	-	0
6.	-	0	0	0	0	2	-1	0	1	0
7.	ND	ND	2	0	4	1	3	0	1	0
8.	0	0	-2	0	-	-2	0	0	1	0
9.	0	0	1	0	1	3	2	0	-	0
10.	0	0	2	0	3	2	1	0	1	0
11.	ND	ND	0	0	2	1	0	0	0	0
12.	0	0	-1	0	1	2	0	0	-	-
13.	-	-	-	0	-	1	-	0	-	-
14.	-	0	0	1	3	1	0	2	1	0
15.	0	0	1	0	4	1	2	0	1	0
16.	-	0	-	0	-	1	-	0	-	0
Mean	0	-0.33	0.55	0	2.55	1.25	1.25	0.53	1	0.08
SE	0	0.41	0.37	0.09	0.41	0.26	0.43	0.25	0.16	0.07

The expression of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 was scored from 0 to 5 as described in the Materials and Methods. Epi = epidermis; Tum = tumor; - = could not be calculated; SE = standard error; ND = not done.

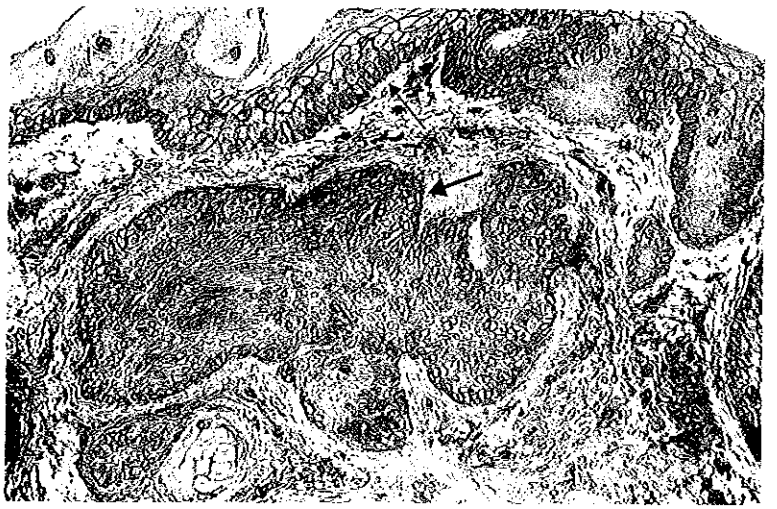

Figure 4: Cryostat section of BCC cultured for 48 h with 900 U/ml rHuIFN-γ stained for CD40. Expression of CD40 can be seen on the tumor cells (fat arrow) and in the epidermis (thin arrow).

Levels of IL-10 in the supernatants

Table III shows the IL-10 levels in the supernatants of the BCC and the normal skin biopsies from BCC patients after culturing for 48 h with and without rHuIFN-γ. The IL-10 levels in the supernatants of the BCC biopsies (n_2) were significantly higher than those in the supernatants of the corresponding normal skin biopsies (n_1) from the same patients ($p=0.00067$, $n_1=10$, $n_2=12$, MWT). The latter were comparable with those from normal (control) skin (results not shown). There was no correlation between the IL-10 levels in the supernatants and the degree of inflammatory infiltrate in both the BCC ($r_s=0.19$, $p=0.55$, $n=12$, SRCT) and the normal skin biopsies from BCC patients ($r_s=0.18$, $p=0.62$, $n=10$, SRCT). Culturing with rHuIFN-γ for 48 h significantly reduced the IL-10 levels in the supernatants of both the BCC ($p\leq0.01$, $n=12$, WT) and the normal skin biopsies from BCC patients ($p=0.01$, $n=10$, WT). The IL-10 levels in the supernatants of the BCC biopsies after culturing for 48 h with rHuIFN-γ (n_2) were not significantly higher than those in the supernatants of normal skin biopsies from BCC patients cultured without rHuIFN-γ (n_1) ($p=0.36$, $n_1=10$, $n_2=12$, MWT). This indicated that culturing with rHuIFN-γ reduces the level of IL-10 practically to normal.

Table III: Levels of IL-10 (pg/ml) in the supernatants of BCC and normal skin biopsies from the same patients after culturing for 48 hours with and without 900 U/ml rHuIFN-γ.

Sample No.	Supernatants of BCC biopsies		Supernatants of normal skin biopsies	
	- rHuIFN-γ	+ rHuIFN-γ	- rHuIFN-γ	+ rHuIFN-γ
1.	188.0	31.9	10.0	0
2.	17.4	5.4	0	0
3.	69.3	73.4	17.5	16.4
4.	51.0	3.8	9.5	0
5.	402.0	311.1	33.0	0
6.	67.0	0	2.7	0
7.	16.4	17.7	7.0	0
8.	155.0	0	10.0	0
9.	37.0	20.4	1.9	2.1
10.	326.8	50.6	30.5	4.5
11.	29.5	19.5	ND	ND
12.	189.0	8.0	ND	ND
Mean	129.0	45.3	12.2	2.3
SE	35.2	23.9	3.4	1.6

- = without; + = with; SE = standard error; ND = not done.

DISCUSSION

BCC of the skin may escape immune surveillance by several mechanisms. First, it has been shown that MHC class I & II antigens, ICAM-1, CD40 and CD80, which are essential for an effective cell-mediated immune response, are either completely lacking or inconsistently expressed on BCC cells [7,9,13-18]. Second, the shedding of ICAM-1 as a soluble form into the surrounding stroma was recently reported [22]. Soluble ICAM-1 may then interact with peritumoral T cells and may inhibit T cell-tumor cell interaction [24]. Third, IFN-γR expression on the BCC cells is down-regulated [22] and fourth, increased expression of immunosuppressive cytokines such as TGF-β [25] and IL-10 [20] have been reported in BCC.

In the present study, the induction and/or up-regulation of HLA-ABC, HLA-DR, ICAM-1, CD40 and CD80 were investigated in BCC biopsies. Using a MAb against HLA-ABC,

47% of the tumors showed weak and diffuse staining for MHC class I similar to that reported in other studies using different MAbs [14,15]. A relatively high dose of rHuIFN-γ failed to up-regulate the HLA-ABC expression on BCC cells. In this and a recent study [22], we showed that BCC cells are able to respond to rHuIFN-γ, since ICAM-1 expression was induced on the BCC cells after culturing BCC biopsies with rHuIFN-γ. We also observed that the induction of HLA-DR expression on normal epidermal keratinocytes required both an increased dose of rHuIFN-γ and culture time compared with those required for ICAM-1 induction. This is in agreement with the results reported by Griffiths et al [10], who observed different responses to IFN-γ for ICAM-1 and HLA-DR induction. The expression of HLA-DR in BCC was investigated in several studies, in which the results were inconsistent. Its expression was not observed on BCC cells in one study [14]. However, it was reported to be expressed on BCC cells in two other studies [13,15], in which HLA-DR expressing tumor cells were observed in close apposition to HLA-DR expressing infiltrate cells. This may indicate the presence of local pro-inflammatory cytokines, which may have induced the expression of HLA-DR on the BCC cells. It is also possible that the cells expressing HLA-DR within the tumor nests were tumor infiltrating T cells, which were difficult to distinguish from the tumor cells. In the present study, it was observed that the cells expressing HLA-DR within the tumor nests after culturing for 48 h with rHuIFN-γ were indeed CD3-positive (Fig. 3), indicating that these cells were activated T lymphocytes. These results suggest that BCC cells have an intrinsic inability to express HLA-DR.

CD40 expression on keratinocytes has been shown to play an important role in costimulation of T cell proliferation [26]. To our knowledge, CD40 expression in BCC has been reported in only two studies with inconsistent results. Wrone-Smith et al [7] reported a weak to moderate cytoplasmic expression of CD40 in tumor nests of BCC, but without any expression in the normal epidermis. In contrast, using a different MAb, Viac et al [9] reported strong expression of CD40 in the basal layer of the epidermis overlying the tumor nests and a drastic loss of expression in the tumor nests. The results of the present study using a MAb that was different from those used in the above two studies corroborate the findings of the latter. In our study, culturing with rHuIFN-γ did not significantly increase the CD40 expression on the BCC cells, although its expression was sporadically observed in a few cases. However, a highly significant increase in CD40 expression was observed in the epidermis overlying the tumor nests and in the epidermis of normal skin from BCC patients. At present, it suffices to say that the precise role of CD40 in the defense against BCC warrants additional investigations.

In murine tumor cell lines, it was shown that the co-expression of CD80 (B7.1) and ICAM-1 was necessary to induce an efficient tumor-specific immune response [5] and

MHC-nonrestricted cytotoxicity [27]. It was also shown that human tumor cells expressing B7 after transfection with a B7 retroviral vector induced strong proliferative and cytotoxic T cell responses [28]. In the present study, CD80 expression was not observed on BCC cells. This was also the case after culturing with rHuIFN-γ, indicating that BCC cells were unable to express CD80. However, CD80 was observed to be induced in the overlying epidermis. IL-10, an immunosuppressive cytokine, has been shown to inhibit the expression of MHC class I & II [19] and CD80 [12] in different tumor cell lines. The results of studies on the IL-10 production by normal human keratinocytes are inconsistent [29-31], but its expression was reported in melanoma [30]. The presence of IL-10 mRNA in BCC was reported in a single study in which also IFN-γ mRNA was observed in the peritumoral T cells [20]. However, in another study, IL-10 mRNA was detected in the tumor infiltrating T cells, but not in BCC cells [21]. In our study, high levels of IL-10 were present in the supernatants of all BCC biopsies. These levels were about 12 times higher than those in the supernatants of normal skin biopsies from BCC patients. Both in BCC and in normal skin from BCC patients there was no correlation between the degree of inflammatory infiltrate and the IL-10 levels. Therefore, it seems highly likely that in normal skin biopsies the keratinocytes and in BCC biopsies the tumor cells are responsible for the production of IL-10. Immunohistochemical investigations using a specific MAb against IL-10 to establish the exact source of IL-10 were unsuccessful. Culturing with rHuIFN-γ reduced the IL-10 levels in the supernatants of BCC biopsies practically to normal. Further studies on the effects of IFN-γ and/or IL-10 on transplanted BCC in immunodeficient (SCID) mice may provide a definite answer. At present, it may be concluded that it is highly likely that the production of IL-10 in BCC is directly or indirectly responsible for the complete lack of expression of HLA-DR, ICAM-1, CD40 and CD80 and the inconsistent expression of HLA-ABC on BCC cells in situ and may be a way for escaping immune surveillance.

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Chapter 4

ADHESION MOLECULES IN BASAL CELL CARCINOMA

4.1. *Expression of E-cadherin, α- & β-catenin and $CD44V_6$ and the subcellular localization of E-cadherin and $CD44V_6$ in normal epidermis and basal cell carcinoma.*

CHAPTER 4.1

Expression of E-cadherin, α- & β-catenin and CD44V$_6$ and the subcellular localization of E-cadherin and CD44V$_6$ in normal epidermis and basal cell carcinoma.

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Submitted for publication

SUMMARY

BCC of the skin is a locally invasive, rarely metastasizing epithelial tumor. In the present study, the expression of E-cadherin, α- & β-catenin and $CD44V_6$ in normal epidermis and on BCC cells were investigated using LM. A reduced expression of E-cadherin, α-catenin and $CD44V_6$ on BCC cells was observed compared with the overlying epidermis. IEM was used to investigate whether this decreased expression of E-cadherin and $CD44V_6$ was due to either an absence or down-regulation of specific membrane structures or due to an overall down-regulation of these adhesion molecules in all membrane structures in BCC. The results showed that E-cadherin and $CD44V_6$ were expressed in adherens junctions, desmosomes and complex interdigitating membrane structures both in normal epidermis and in BCC. A quantitative analysis showed that only a percentage of desmosomes was stained, indicating that these adhesion molecules are not essential for maintaining the integrity of these structures. In addition, the effect of pro-inflammatory cytokines, such as IFN-γ and TNF-α, which may be produced by the peritumoral inflammatory infiltrate, was investigated in biopsies of normal skin and BCC using a biopsy culture system and immunohistochemistry. The expression of E-cadherin and $CD44V_6$ was not significantly decreased after culturing BCC or normal skin biopsies for 48 h with or without rHuIFN-γ or rHuTNF-α.
It may be concluded that the decreased expression of both E-cadherin and $CD44V_6$, observed in LM, was not due to the absence of specific specialized structures in BCC and most likely also not due to down-regulation by local cytokines, but rather due to generic down-regulation of both these adhesion molecules during malignant transformation.

INTRODUCTION

Cell-cell adhesion plays an important role in organ morphogenesis, cell growth and differentiation. Desmosomes and adherens junctions are structures, which connect intermediate filaments and actin to the plasma membrane, respectively [1]. Ultrastructurally, adherens junctions are difficult to recognize and were described by Kaiser et al [2] on the basis of the perfectly parallel arrangement of the plasma membranes at the sites of cell-cell contact. They can be distinguished from desmosomes by the absence of associated electron-dense keratin bundles. An important component of adherens junctions is E-cadherin. E-cadherin interacts with catenins through which it is linked to actin [1-4] and plays a key role in keratinocyte intercellular junction

organization and epidermal morphogenesis [3,5]. Functional catenins are required for normal E-cadherin function [1]. Reduced expression of E-cadherin and α- & β-catenin in carcinoma cells is associated with tumor dedifferentiation, increased invasiveness and metastasis development [1,6-11]. Cytokines, such as TNF-α, have been shown to down-regulate the expression of cadherin/catenin complexes, causing dyscohesion of epithelial cells [12].

Adhesion molecule CD44, of which at least ten different variant isoforms have been identified [13], also plays a role in keratinocyte-keratinocyte adhesion [14]. In contrast to E-cadherin and α- & β-catenin, $CD44V_6$ expression was positively correlated with invasive growth and metastasis development in some tumors [15-17]. The expression of CD44 has been shown to be up-regulated by IFN-γ [18].

BCC of the skin is a common, locally invasive, rarely metastasizing epithelial tumor. A possible role for cell-cell adhesion molecules in the growth and aggressive behavior of this tumor has been investigated in several LM studies [19-25]. E-cadherin expression on BCC cells was reported to be especially reduced in the infiltrative type of BCC [22,23]. The expression of $CD44V_6$ in BCC was generally reduced and varied within tumor nests [19-21,24,25]. To our knowledge, the exact ultrastructural localization of E-cadherin and $CD44V_6$ in BCC is still unknown.

The aim of this study was to investigate the expression of E-cadherin, α- & β-catenin and $CD44V_6$ on BCC cells and normal keratinocytes in situ and after culturing biopsies of BCC and normal skin with rHuIFN-γ and/or rHuTNF-α. In addition, the subcellular localization of both E-cadherin and $CD44V_6$ in normal epidermis and BCC was investigated to elucidate whether the decreased expression of both E-cadherin and $CD44V_6$ on tumor cells observed in LM was due to a specific down-regulation of these molecules in specialized membrane structures.

MATERIALS AND METHODS

Immunohistochemistry

Fifteen specimens of BCC, 9 nodular, 2 nodular/adenoid, 2 nodular/superficial and 2 superficial type, were obtained by surgical excision from 13 patients. A biopsy was taken, snap-frozen immediately in liquid nitrogen-cooled isopentane and stored in liquid nitrogen. The diagnosis was confirmed histologically by examination of H&E-stained sections.

Cryostat sections (6 μm) were stained using the immunoperoxidase-streptavidin-biotin complex (ABC) method as described previously [26]. MAbs against E-cadherin (Organon

Technika, Boxtel, The Netherlands) and CD44V_6 (R&D Systems, ITK Diagnostics, Uithoorn, The Netherlands), both IgG_1 isotype, and polyclonal antibodies (PAbs) against α- & β-catenin (kindly provided by Prof. dr. M. Mareel, Gent, Belgium) were used. DAB was used as substrate.
The stained sections were scored as follows: levels of expression of E-cadherin, α -& β-catenin and CD44V_6: 0, 0%; 1, 0-25%; 2, 25-50%; 3, 50-75%; 4, >75% positively stained tumor cells and the staining intensity: -, no staining; +/-, weak diffuse staining; +, diffuse staining; ++, membranous staining comparable with that in the normal epidermis.

Short-term culture
At least 3 biopsies of 3 mm diameter each from 12 BCCs were obtained from 12 patients, 5 males and 7 females, aged 46 to 90 years. Normal (control) skin was obtained from healthy patients undergoing breast reductions. One of the biopsies of BCC and normal skin was immediately snap-frozen in liquid nitrogen and stored at -80°C. The other biopsies were placed in a transwell-culture system (Costar, Badhoevedorp, The Netherlands) and were cultured in IMDM (Gibco, Paisley, U.K.) containing 1% NHS as described previously [27]. After culturing the biopsies for 48 hours with or without rHuIFN-γ (Boehringer Ingelheim, Alkmaar, The Netherlands) and/or rHuTNF-α (Amersham, s'-Hertogenbosch, The Netherlands), they were carefully removed, snap-frozen in liquid nitrogen and stored at -80°C. Cryosections (6 μm) were cut and stained as described above.

Immunoelectron microscopy
Six biopsies of BCC, 5 nodular and 1 nodular/adenoid type, obtained from 5 patients and 3 biopsies of normal skin were fixed in 4% paraformaldehyde overnight at 4°C. Then they were rinsed in PBS, embedded in Tissue Tec (Klinipath, Duiven, The Netherlands) and frozen for cryosectioning. Cryosections of 60 μm were cut and collected in PBS. The sections were then rinsed in 0.5 M ammoniumchloride (NH_4Cl) for 1 h at room temperature to minimize aspecific staining. They were subsequently incubated with optimal dilutions of the above MAbs, peroxidase-conjugated GAM immunoglobulin and DAB as substrate. Then they were rinsed in distilled water and incubated with 1% osmium-potassiumferrocyanide for 1 h, rinsed in distilled water again and subsequently in PBS overnight at 4°C. After a step-wise dehydration in increasing percentages of ethanol they were impregnated with a 1:1 mixture of ethanol and epoxy resin (LX112, Carl Zeiss, Weesp, The Netherlands) for 1 h at room temperature and then with pure epoxy resin for 1 hour at 37°C. Then they were embedded in Beem capsules (Norticon, Breda, The Netherlands) and polymerized at 60°C for a minimum of 12 hours. Ultrathin sections (±40

nm) were cut and examined without counter-staining in a Zeiss Electron Microscope 902 (Carl Zeiss, Weesp, The Netherlands) operated at 80 kV. The percentages of stained membrane structures were determined at a magnification of 30,000 by counting the total number of specific structures and the number of structures that were positively stained in the same area. Negative controls included the omission of primary antibody and a MAb against CD20 (Becton Dickinson, Erembodegem-Aalst, Belgium), IgG_1 isotype, was used as isotype control at identical protein concentrations.

Statistical analysis

The results were analyzed using the WT. The level of significance, representing two-tailed testing, and the number of samples (n) are mentioned in the Results. A p value of lower than 0.05 was considered to be statistically significant.

RESULTS

Light microscopy

The results of immunohistochemistry are summarized in Table I. Both normal epidermis and the epidermis overlying the BCC cells showed similar membrane expression of E-cadherin and $CD44V_6$ in the stratum basale and the stratum spinosum. No expression of either of these molecules in the stratum granulosum and the stratum corneum was observed. α-catenin was detected on the membranes of the upper part of the stratum spinosum and the stratum granulosum in both normal and overlying epidermis. However, the stratum basale and the lower part of the stratum spinosum showed a more diffuse staining. Strong staining of the membranes in all layers of both normal and overlying epidermis was observed for β-catenin.

More than 70% of the BCC cells showed E-cadherin expression in all the examined biopsies. In only 2 (13.3%) of the 15 samples the staining of the tumor cells was weak and diffuse compared with the overlying epidermis (Fig. 1). In 14 (93.3%) of the 15 samples all BCC cells were stained for α-catenin, although the staining was less intense compared with the overlying epidermis (Fig. 2a). β-catenin was observed to be expressed in all BCC cells in all the examined biopsies. In 11 (73.3%) of the 15 samples strong membrane staining of the BCC cells was observed (Fig 2b) and only 1 (6.7%) of the 15 tumors showed weak and diffuse staining for β-catenin. $CD44V_6$ expression on BCC cells was considerably more heterogeneous compared with E-cadherin and α- & β-catenin expression. The percentage of $CD44V_6$ positive tumor cells varied between 0% and 100%. No correlation was found between the percentage positive tumor cells and the

histopathological types of the BCC. Representative examples of the staining pattern are shown in Fig. 3a & b. The staining intensity varied between no staining at all to strong membrane staining of the tumor cells.

Table I: The expression of E-cadherin, α- & β-catenin and $CD44V_6$ on BCC cells.

E-cadherin		α-catenin		β-catenin		$CD44V_6$	
Expr.	Int.	Expr.	Int.	Expr.	Int.	Expr.	Int.
4	++	4	+/-	4	+	4	+
4	+	4	+/-	4	++	1	+/-
3	+	4	+/-	4	+	1	+
4	++	4	+	4	++	3	+
4	++	4	+/-	4	+/-	1	+
4	++	4	++	4	++	4	++
4	++	4	+/-	4	++	3	+/-
4	++	4	+	4	++	1	++
4	++	4	+	4	++	0	-
4	+/-	4	+	4	++	4	+/-
4	+/-	4	+/-	4	++	1	+
3	+	4	+/-	4	++	1	+/-
4	++	4	+	4	++	2	+/-
3	++	4	+	4	+	4	+
4	++	4	+/-	4	++	1	++

The levels of expression (Expr.) and the intensity (Int.) of the staining for the adhesion molecules are scored as described in the Materials and Methods.

Short-term culture

Normal skin and BCC biopsies were cultured for 48 h with doses of rHuIFN-γ and/or rHuTNF-α varying from 0 to 1200 U/ml. The expression of E-cadherin and $CD44V_6$ on normal keratinocytes and BCC cells after culturing with rHuIFN-γ and/or rHuTNF-α was not significantly different from that observed after culturing without these cytokines (n=12, WT). (Results not shown)

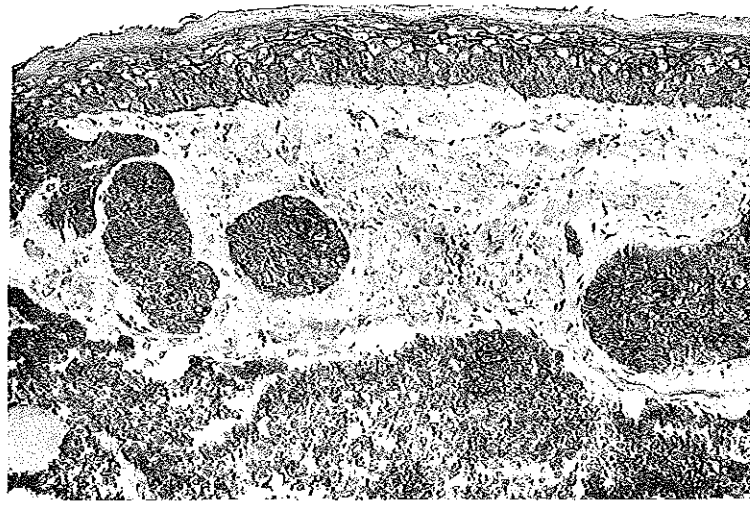

Figure 1: Nodular type of BCC stained for E-cadherin, x250. Staining of the tumor cells is diffuse compared with the overlying epidermis.

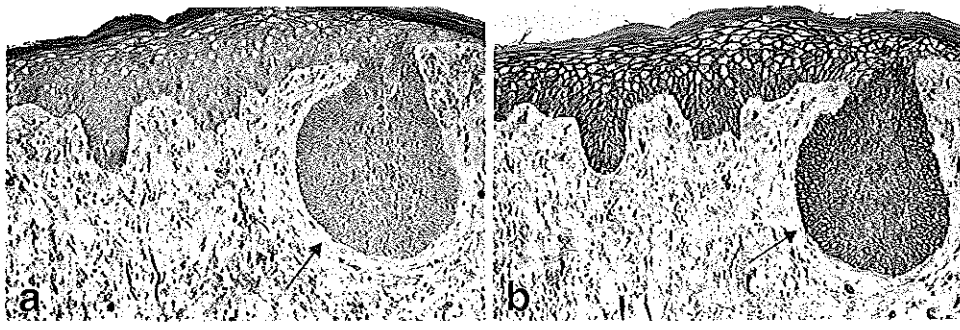

Figure 2: Cryosections of superficial type of BCC stained for α-catenin (a) and β–catenin (b), x 400; a, the staining of the tumor cells is weak and diffuse (arrow) compared with the overlying epidermis; b, both the tumor cells (arrow) and the overlying epidermis show strong membrane staining

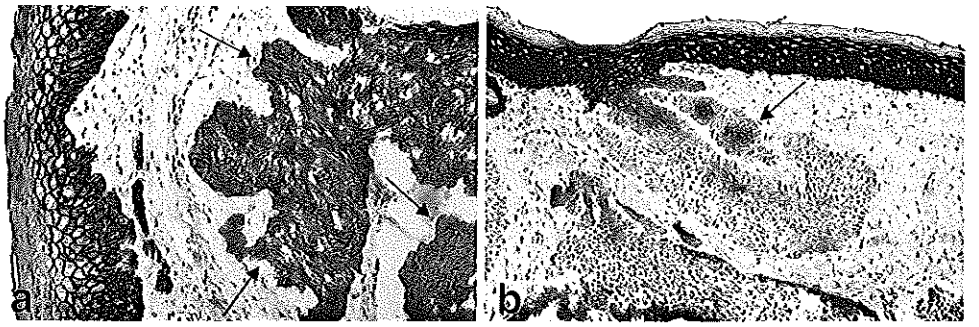

Figure 3: Cryosections of nodular (a) and nodular/superficial (b) type of BCC stained for $CD44V_6$, x 250; a, all tumor cells are stained (arrows); b, only a small proportion of the tumor cells are stained (arrow).

Immunoelectron microscopy

In normal epidermis, the basement membrane, stratum corneum and stratum granulosum completely lacked immunolabelling for both E-cadherin and $CD44V_6$. However, varying proportions of desmosomes, complex interdigitating membrane structures and adherens junctions were stained for both molecules. The percentages of E-cadherin and $CD44V_6$ positive desmosomes in normal epidermis were highly reproducible and are shown in Table II. The mean percentage of E-cadherin positive desmosomes in normal epidermis ranged from 12.6% in the upper third to 69.3% in the lower third of the stratum spinosum. However, only 57.9% of the desmosomes in the basal layer were stained. Representative examples of the staining pattern of the desmosomes and the adjacent cell membrane structures are shown in Fig. 4a & b. The percentage of $CD44V_6$ positive desmosomes in normal epidermis ranged from 37.8% in the upper layer of the stratum spinosum to 65.5% in the basal layer. An example of the staining pattern is shown in Fig. 5a. The interdigitations, representing complex structures of cell-cell contact as shown in Fig. 5b, were most prominent in the stratum basale and decreased in size and staining intensity towards the upper layers. The percentage of stained adherens junctions is difficult to determine, because the unstained junctions are difficult to identify [2]. In BCC, membrane structures similar to those in normal epidermis were stained for both E-cadherin and $CD44V_6$ (Fig. 6a & b, respectively). The percentages of the stained desmosomes and interdigitations in BCC are shown in Table III. The mean percentage of the E-cadherin positive desmosomes (53.8%) was higher than that of $CD44V_6$ positive desmosomes (32.6%) in BCC. However, the mean percentage of E-cadherin positive interdigitations (35.3%) was lower

than that of those expressing $CD44V_6$ (45.8%). Not all BCC cells were stained for $CD44V_6$ and the mentioned percentages of $CD44V_6$ expressing specialized membrane structures were based only on stained BCC cells. No immunoreactivity in normal epidermis and in BCC was observed when an IgG1 isotype control antibody replaced the E-cadherin or CD44V6 specific MAb.

Figure 4: Electron micrographs of normal epidermis stained for E-cadherin. Both the desmosomes (thin arrows) and the adjacent membrane structures (arrowhead) were stained. Also negative desmosomes were observed (b, fat arrow). The desmosomes can be easily recognized by the presence of associated keratin bundles (a, asterix). Bar = 0.6 μm.

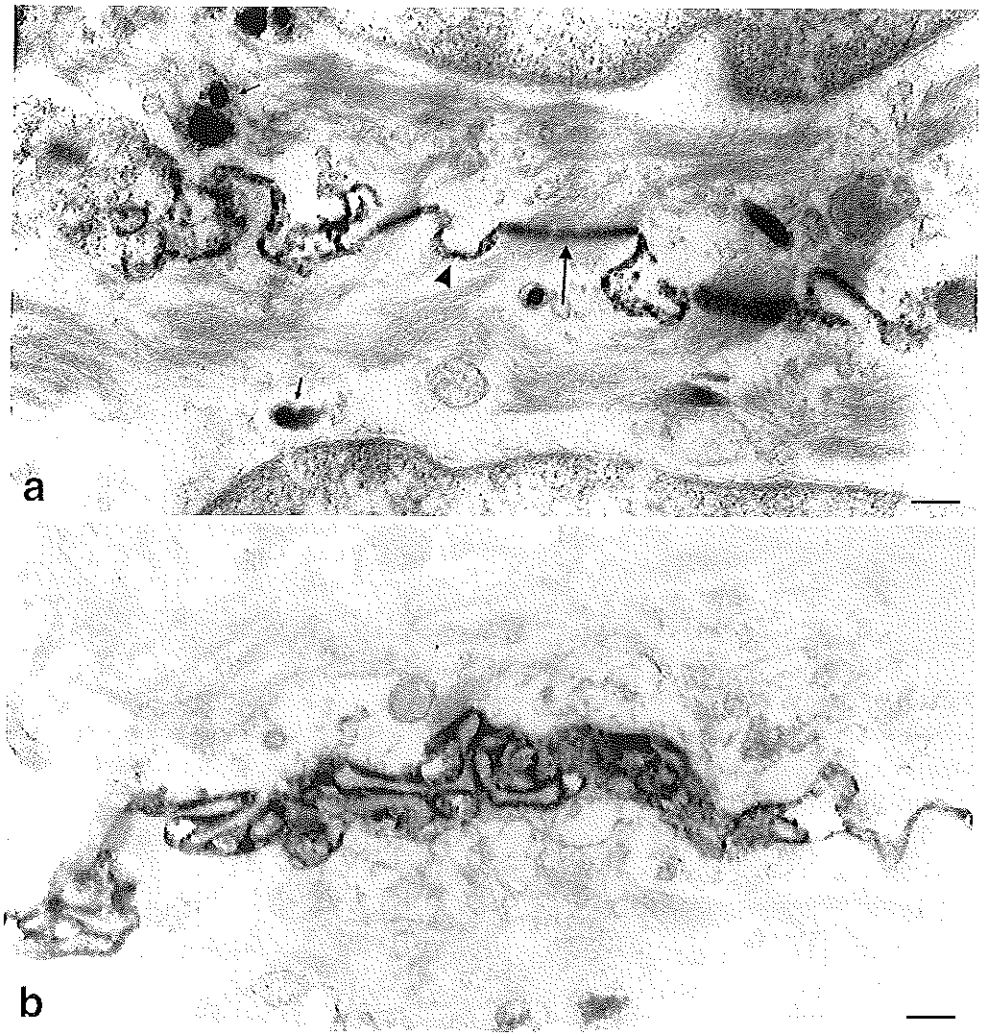

Figure 5: Electron micrographs of basal keratinocytes, indicated by the presence of melanosomes (small arrows) in the normal epidermis, stained for $CD44V_6$. a, both the desmosomes (arrow) and adjacent structures (arrowhead) were stained; b, complex membrane interdigitation strongly positive for $CD44V_6$. Bar = 0.6 μm.

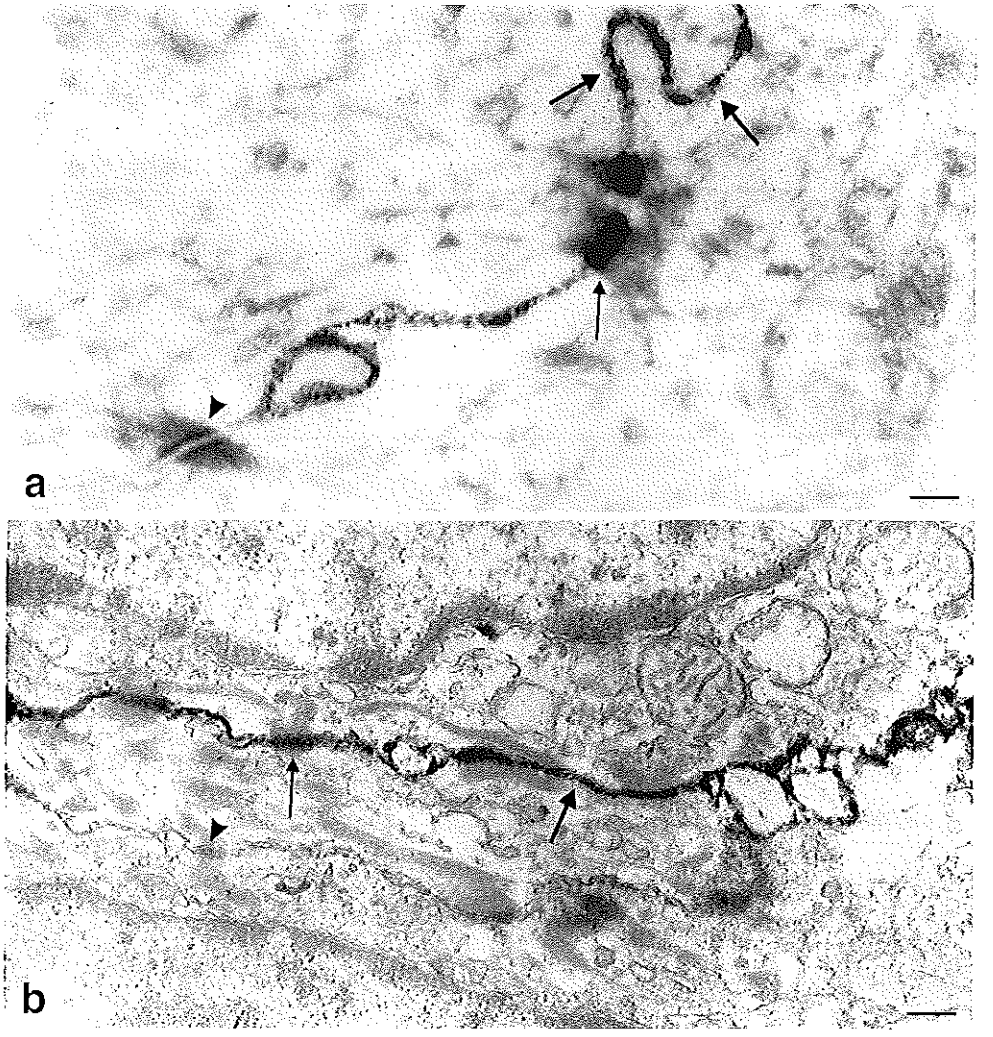

Figure 6: Electron micrographs of membrane structures in BCC stained for E-cadherin (a) and CD44V_6 (b). Both stained (thin arrows) and negative desmosomes (arrowheads) were observed. Structures, which may be described as adherens junctions also express both molecules (fat arrows).

Table II: Percentages of stained desmosomes in normal epidermis.

Epidermal layer	E-cadherin (n=3)	$CD44V_6$ (n=2)
I	12.6	37.8
II	43.2	56.7
III	69.3	58.7
IV	57.9	65.5

I = Upper third part of the stratum spinosum; II = Central part of the stratum spinosum; III = Lowest third part of the stratum spinosum; IV = Stratum basale.

Table III: Percentages of stained desmosomes and interdigitations in BCC.

Sample	Desmosomes		Interdigitations	
No.	E-cadherin	$CD44V_6$	E-cadherin	$CD44V_6$
1.	55.7	4.8	48.0	35.4
2.	ND	36.4	ND	32.0
3.	48.2	ND	49.0	ND
4.	66.6	48.7	0	42.8
5.	47.7	13.9	50.0	66.6
6.	50.8	59.2	29.4	52.3
Mean	53.8	32.6	35.3	45.8
SE	3.5	10.3	9.6	6.3

ND = not done; SE = Standard error.

DISCUSSION

The expression of E-cadherin, α- & β-catenin and $CD44V_6$ in both normal epidermis and on BCC cells was investigated at LM level and E-cadherin and $CD44V_6$ at ultrastructural level in the present study. E-cadherin and $CD44V_6$ were observed in all viable layers of the normal epidermis, except in the stratum granulosum and on the dermal side of basal cells, corroborating the results of previous LM studies [22,24,28]. Others, however, did

detect $CD44V_6$ expression in the stratum granulosum [19-21]. Haftek et al [3], described the presence of vinculin, which is homologous to α-catenin, and β-catenin in all viable layers of the epidermis. The staining for vinculin was the strongest in the upper part of the stratum spinosum and stratum granulosum, corroborating our results.

The ultrastructural localization of E-cadherin, known as a component of adherens junctions [1,3,4] was described both in keratinocytes [3] and in intestinal epithelium [28]. Horiguchi et al [28], reported that besides adherens junctions, E-cadherin was also expressed in desmosomes of normal keratinocytes. This was in contrast with that reported by Haftek et al [3]. In our study using a pre-embedding peroxidase immunolabelling technique, comparable with that used by Horiguchi et al [28], E-cadherin was observed in adherens junctions, desmosomes and complex interdigitating cell membrane structures. Haftek et al [3] used a post-embedding technique and immunogold labeling. The lower sensitivity of their technique may well explain the differences between their results and those reported here.

Quantitative analysis showed a decreased percentage of E-cadherin expressing desmosomes from the lowest to the upper layer of the stratum spinosum in normal epidermis. This finding suggests that down-regulation of E-cadherin expression in desmosomes is associated with terminal differentiation of normal epidermal keratinocytes. $CD44V_6$ appeared to be expressed in similar subcellular structures as E-cadherin. In addition, a decrease in $CD44V_6$ expressing desmosomes from the basal layer to the superficial layers was observed. This finding corroborates the observations by Seiter et al [19], who showed a gradient of CD44 expression and its splice variants at LM level. The decrease in CD44 expression was previously observed to correlate with terminal differentiation of keratinocytes [14]. The fact that only a proportion of the desmosomes was stained for E-cadherin and $CD44V_6$ indicates that expression of these molecules is not a prerequisite for maintaining the integrity of the desmosomal structure.

Skerrow et al [29] reported an increase in desmosome frequency from a minimum in the basal layer towards a maximum in the granular layer. Combining their observations with ours, it may be stated that the decrease in staining intensity for $CD44V_6$ and E-cadherin in the upper layers of the normal epidermis observed at LM level was not due to a reduced number of desmosomes, but due to the diminished percentage of desmosomes and other specialized membrane structures expressing both these molecules. Although we did not quantitate the percentage of immunostained adherens junctions and interdigitations, it seemed that there was also a decrease in staining for these adhesion molecules in these structures in the upper part of the normal epidermis.

At LM level, we observed a decreased expression of E-cadherin and α-catenin on BCC cells compared with the overlying epidermis similar to that observed in other studies

[22,23,30]. This is in agreement with the fact that reduced E-cadherin expression is associated with tumor de-differentiation and increased invasiveness [10]. BCC has a de-differentiated phenotype as was shown by the presence of simple epithelial Ck such as Ck 7,8,18 and 19 [26] and although metastasis is rare in BCC, there is definitely invasive growth. In addition, preserved expression of β-catenin was observed in this study. The expression of $CD44V_6$ was heterogeneous and strongly down-regulated on BCC cells compared with the overlying epidermis, corroborating the findings in several studies [19-21,24,25].

The expression of E-cadherin and $CD44V_6$ has been shown to be influenced by pro-inflammatory cytokines such as TNF-α and IFN-γ [12,18]. Since varying degrees of inflammatory infiltrate, mainly consisting of T cells [31] surrounds the tumor nests of BCC, the down-regulation of E-cadherin and $CD44V_6$ may be caused by the local presence of the cytokines. In the present study, incubation of biopsies of normal skin and BCC with relatively high doses of rHuIFN-γ and rHuTNF-α or combinations of these did not significantly alter the expression of E-cadherin and $CD44V_6$ on keratinocytes of normal epidermis or on BCC cells. However, the expression of ICAM-1 was up-regulated, indicating that the epidermal and BCC cells were able to respond to rHuIFNγ as reported previously [27]. Therefore, it is unlikely that the presence of IFN-γ and TNF-α in situ plays a role in the down-regulation of E-cadherin and $CD44V_6$ on the BCC cells.

Similar to that in the normal epidermis, in BCC, E-cadherin and $CD44V_6$ were localized in desmosomes, adherens junctions and interdigitations, indicating that the decreased expression of both these molecules observed in BCC at LM level was not due to the loss of a specific subcellular structure, but was suggestive of a general down-regulation in their expression. The mean percentage of E-cadherin positive desmosomes in BCC cells was higher than that of $CD44V_6$ positive desmosomes, whereas the percentage of $CD44V_6$ expressing complex interdigitations was higher than the percentage of those expressing E-cadherin.

In contrast to E-cadherin, $CD44V_6$ expression was positively correlated with tumorigenesis and the development of metastasis in colon and breast carcinomas [15,16]. However, in NMSC such a correlation was not observed. In these cancers, $CD44V_6$ expression on tumor cells was shown to be reduced compared with that on normal keratinocytes [19,20,25]. In SCC of the skin, down-regulation of $CD44V_6$ was described to correlate with a higher degree of differentiation [24]. This is analogous with our findings in normal epidermis in which the upper layers showed a reduced expression of $CD44V_6$. However, in BCC, $CD44V_6$ does not appear to correlate with the degree of differentiation. In fact, BCC represents a poorly differentiated phenotype, but nevertheless shows decreased expression of $CD44V_6$.

In the present study, it was observed that both E-cadherin and $CD44V_6$ were expressed in all subcellular structures related to cell-cell adhesion both in normal epidermis and in BCC. In particular, the hitherto less prominent role of complex interdigitations in cell-cell adhesion is emphasized by their intense labeling for E-cadherin and $CD44V_6$. However, the expression of neither E-cadherin nor $CD44V_6$ was obligatory in any of these structures. The structures expressing both these molecules in BCC were similar to those in normal epidermis. It was also observed that the decrease in expression of these molecules was not caused by the presence of pro-inflammatory cytokines such as IFN-γ and TNF-α. This allows the conclusion that the decreased staining for E-cadherin and $CD44V_6$ in BCC observed at LM level is not related to the absence or down-regulation of specialized membrane structures or due to the presence of pro-inflammatory cytokines, but because of a non-specific lowering in their expression. Whether this is in any way related to the non-metastatic behavior of this tumor still remains to be established.

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Chapter 5

SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

In the studies described in this thesis, three different aspects of BCC were investigated, namely; 1) The expression of Cks; 2) The immunological defense against this tumor, and; 3) The expression of some of the adhesion molecules generally involved invasive growth and metastasis of epithelial tumors. Investigations into these aspects may contribute to a better insight into the pathogenesis and pathophysiology of BCC.

Cytokeratin expression

Since the exact origins of the various types of BCC still remain unknown and because patterns of Ck expression are often preserved after tumor cell transformation, many investigations have focussed on the expression pattern of epithelial Cks in BCC [1-8]. These investigations were also undertaken in the hope that a suitable histopathological tumor marker for BCC might be identified. Such a specific tumor marker would be of significant practical value in the determination of surgical margins [9], either in a conventional setting or as part of a MMS procedure [10]. Epithelial Cks 8, 18 and 19 have been used as tumor markers to identify metastases of small cell lung cancer [11,12] and SCC of the lung [13]. In healthy human skin these Cks are expressed in the sweat glands and sebaceous glands and the outer root sheath of the hair follicle, but they are not expressed in the epidermis. Conflicting results on the expression of Cks 8, 18 and 19 in BCC have been reported in previous studies [1-8]. Therefore, we investigated their expression in BCC, using a large panel of MAbs against these Cks. The results are described in **chapter 2.**

In the study described in **chapter 2.1**, 23 BCCs were investigated, using a panel of 13 different MAbs against epithelial Cks 7, 8, 18, 19 and one against the high molecular weight Ck 10. Expression of Ck 8 was detected in all 23 BCCs with MAb 4.1.18. Two other MAbs showed inconsistent staining of Ck 8 and a third one did not show any staining at all. (**Table II, 2.1**). Cks 7 and 19 were detected inconsistently, whereas Cks 18 and 10 were not detected in any of the BCCs. In the study described in **chapter 2.2** we investigated, whether the observed conflicting differences in the staining for Ck 8 described in **chapter 2.1** may have been caused by variations in the technique. Therefore, the same MAbs against Ck 8 were used in a comparative LM and IEM study. The results of this study confirmed the staining patterns observed in the LM investigations (**Table II, 2.2**). Since only MAb 4.1.18 showed consistent staining for Ck 8 in all samples in both studies (**2.1 and 2.2**), it was used to detect Ck 8 as a marker for BCC cells in the subsequent investigations, described in this thesis.

Recently, Krekels et al [14] reported expression patterns of Cks in recurrent and primary BCC. Those authors observed that the expression of Cks 5, 14 and 17 was similar in both types, whereas simple epithelial Cks 8 and 19 were increased in the recurrent BCCs compared with the corresponding primary tumors. The expression was predominantly located at the tumor invasion fronts. However, Krekels et al only used anti-Ck 8 MAb M20, but did not use MAbs 4.1.18 and 35βH11, which in our study stained a high percentage of BCC cells (**chapter 2**). These latter two MAbs were also used in other investigations into the expression of Ck 8 in SCC [15] and sebaceous carcinoma [7,16]. In the first study [15], well differentiated SCC were not stained with MAb 4.1.18, whereas only some tumor cells were stained in poorly differentiated SCC. MAb 35βH11 did not stain any of the 35 investigated SCCs. Therefore, these MAbs were suggested to be useful in distinguishing BCC from SCC [15]. However, one should be very cautious indeed in using Cks as specific tumor markers. The latter studies [7,16] reported the expression of Ck 8 using MAb 35βH11 in about 80% of the sebaceous carcinomas, but in none of the BCC and SCC examined. The authors suggested that the use of MAb 35βH11 may be useful immunohistochemically for ruling out both SCC and BCC in the differential diagnosis. However, the results of our studies (**chapter 2**) are clearly at variance with these reports. In addition, they illustrate that the use of different MAbs against the same Ck may lead to significant differences in results obtained. Therefore, the use of more than one MAb directed at any one Ck to investigate the Ck expression is strongly recommended. It would appear possible, however, to use a MAb against a specific Ck as an adjunct in the evaluation of surgical margins or to locate tumor cells amidst a dense inflammatory infiltrate. The differences in staining between the different MAbs against Cks are most probably caused by differences of epitopes, detected by the antibodies. Recently, detailed information on the epitopes recognized by different MAbs against Cks 8, 18 and 19 have been reported [17]. In Cks 8 and 19, at least 2 closely related major immunogenic epitopes are identified, whereas 4 different epitopes of Ck 18 are noted. This indicates that selective epitope specificities of Cks 8, 18 and 19 can be detected. This is of obvious importance in immunohistochemical investigations.

The exact origin of BCC still remains unknown, but on the basis of Ck expression pattern different suggestions were made [7,8]. It may be possible that different types of BCC have different origins such as the outer root sheath of the hair follicle (nodular and/or adenoid type) and the basal layer of the epidermis (superficial type). Further investigations are necessary to characterize the cell type (or all types) of origin.

The immune response against basal cell carcinoma

The tumor nests of BCC are surrounded by varying degrees of inflammatory infiltrate. This infiltrate consists mainly of T cells and a small number of macrophages, LC, NK cells and B cells, indicating a T cell-mediated immune response [18]. The T cells are predominantly $CD4^+$ with a CD4/CD8 ratio of ±2.0, which is a common feature of inflammatory infiltrates associated with solid tumors [19]. A proportion of the T cells is activated, because they express HLA-DR [20], CD25, CD45RO and the transferrin receptor [21]. Despite the presence of these activated T cells the cell-mediated immune response against BCC would appear to be ineffective. In this respect, it is of interest that even though a few cases of spontaneous tumor regression have been reported [22], hardly any T cells are present within or in close apposition to the tumor nests. In an effective T cell-mediated immune response the T cell-tumor cell interaction, which is comparable with the interaction between a T cell and an APC, is of crucial importance for the T cell activation. This interaction requires several molecules on the T cell and the tumor cell (Table I).

Table I: Molecules that play a pivotal role in T cell-tumor cell interaction.

Tumor cell (APC)	T cell
MHC class I	T cell receptor (TCR)
MHC class II	
ICAM-	LFA-1
CD40	CD40 Ligand
CD80 (B7.1)	CD28

BCC cells are known not to express ICAM-1 and HLA-DR, whereas MHC class I expression is inconsistent [23-26]. The expression of CD40 was reported to be down-regulated on BCC cells [27,28], and to our knowledge, CD80 expression in BCC has not yet been investigated. The lack or down-regulation of these molecules on BCC cells may preclude T cell-tumor cell interaction and an effective immune response. Pro-inflammatory cytokines such as TNF-α and IFN-γ can induce these molecules on epithelial cells, including keratinocytes [29,30], whereas the immunosuppressive cytokine IL-10 may inhibit their expression [31].

The studies reported in **chapter 3** focus on the lack of an effective T cell-mediated immune response against BCC. In **chapter 3.1**, we report on a transwell-culture system in

which tumor biopsies could be cultured for at least 48 hours. The system was optimized and used to investigate rHuIFN-γ-induced expression and shedding of ICAM-1 in BCC and whether that correlated with the IFN-γR expression. We found that both ICAM-1 expression on the BCC cells (**Table I, 3.1**) and the level of sICAM-1 in the culture media (**Fig 4, 3.1**) were significantly up-regulated by rHuIFN-γ. The induction of ICAM-1 on the epidermis overlying the tumor nests was significantly higher than that on the BCC cells. This could have been caused by differences in the expression of the IFN-γR, since immunohistochemistry showed a significant down-regulation of these receptors on the tumor cells (**Table II, 3.1**). These results allow the conclusion that down-regulation of IFN-γR expression in combination with the shedding of ICAM-1 may be the way by which a local T cell-mediated immune response is evaded in BCC.

In the study described in **chapter 3.2**, the induction and/or up-regulation of MHC class I & II antigens, CD40 and CD80 by rHuIFN-γ were investigated. In addition, the level of IL-10 in the culture supernatants was determined. The results showed that in the tranwell-culture system under optimal culture conditions, in the epidermis only CD40 and ICAM-1 were significantly up-regulated, whereas a trend was noted in the up-regulation of HLA-DR and CD80. On the BCC cells, a significant up-regulation of ICAM-1 expression was observed and CD40 expression was only slightly up-regulated. The expression of MHC class I, HLA-DR and CD80 in BCC biopsies was not altered by rHuIFN-γ (**Table II, 3.2**). The levels of IL-10 were significantly increased in the culture supernatants of tumor biopsies compared with normal skin (**Table III, 3.2**), indicating the presence of IL-10 in BCC in vivo. Kim et al [32] observed high levels of IL-10 mRNA in BCC cells and high levels of IL-10 in culture media of BCC cells, indicating that IL-10 was most likely produced by BCC cells. These findings may contribute towards a better understanding of the lack of an effective T cell-mediated immune response in BCC, despite the presence of peritumoral T cells. The absence or down-regulation of MHC class I & II antigens on BCC cells may preclude effective T cell-tumor cell interaction. Indeed, in those instances where this interaction does take place, the absence of costimulatory molecules on BCC cells may lead to T cell anergy [33]. In an anergic state the T cells are refractory to stimuli and unable to produce cytokines such as IFN-γ, TNF-α, IL-12, IL-2, etc. The production of IL-10 by BCC cells may also induce T cell anergy [34]. In addition, IL-10 has been shown to inhibit growth of tumor reactive T cells [35].

To summarize, the following mechanisms have been identified in BCC that may preclude an effective T cell-mediated immune response against these tumors (Fig 1);

* Inconsistent expression of HLA-ABC (MHC I) and complete lack of HLA-DR on BCC cells *(1)*.
  Absence of the costimulatory molecules, ICAM-1, CD40 and CD80, on BCC cells *(1)*, inducing T cell anergy after TCR-MHC interaction *(2)*.
* Production of IL-10 by the BCC cells *(3)*, which may be responsible for the complete lack of HLA-DR, ICAM-1, CD40 and CD80 and the inconsistent expression of HLA-ABC *(4)* on BCC cells and the induction of T cell anergy *(5)*.
* Lack or low production of pro-inflammatory cytokines, such as IFN-γ by T cells due to inhibition by IL-10 *(6)*.
* Down-regulation of IFN-γR on BCC cells.
* Shedding of ICAM-1 into the surrounding stroma which may trap the T cells and prevent T cell-tumor cell interaction *(7)*.

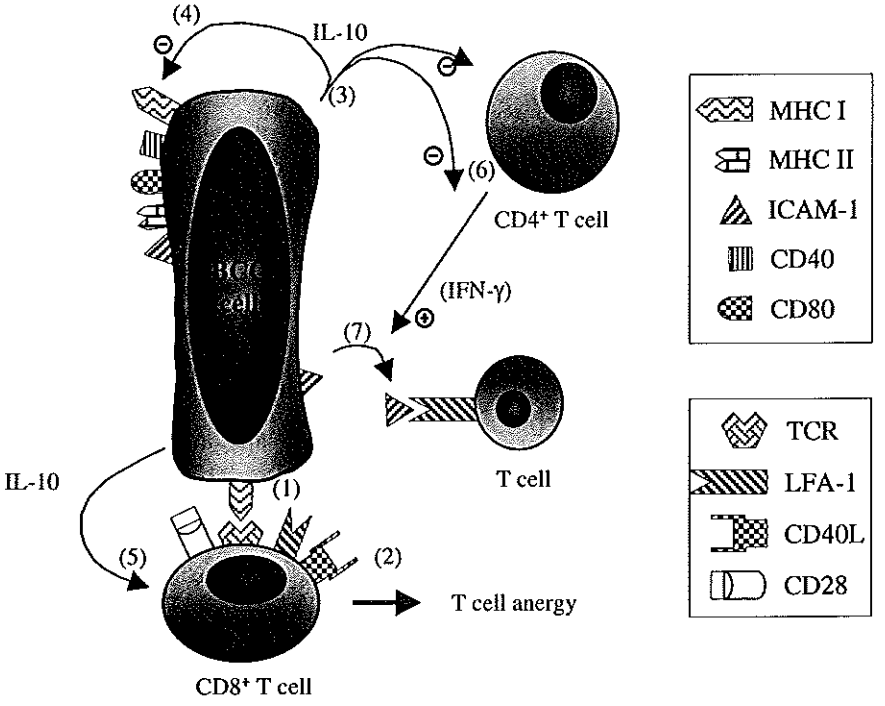

Figure 1: Mechanisms in BCC that may contribute to the lack of an effective T cell-mediated immune response against the tumor.

Adhesion molecules and metastasis

Although BCC is a malignant tumor capable of invasive growth and it has the potential to cause extensive destruction of underlying tissues including bone, the percentage of metastasis is very low. Indeed, only several cases have been reported so far [36-38]. In invasive growth and metastasis formation, adhesion molecules, such as E-cadherin and CD44 have been described to play an important role in carcinomas of the breast [39,40] and colon [41]. These molecules are involved in cell-cell adhesion, and in normal skin they are expressed on the cell membranes in the viable epidermal layers. E-cadherin has been identified in adherens junctions, where it plays a role in cell-cell contact via homotypical interaction. It is connected to the actin cytoskeleton through binding with α–, β– and γ-catenin. Functionally intact catenins are required for normal E-cadherin function [42]. The exact localization of $CD44V_6$, to our knowledge, has so far remained elusive.

The expression of E-cadherin, α- and β-catenin and $CD44V_6$ at LM level was investigated in the study reported in **chapter 4**. This study was pursued in order to obtain a better insight into the non-metastatic behavior of BCC. The results showed that the expression of E-cadherin, α-catenin and $CD44V_6$, but not of β-catenin, was down-regulated on the tumor cells compared with the overlying epidermis. It has become more clear that β-catenin plays a role in signal transduction and stimulation of the expression of a number of genes involved in apoptosis and cell proliferation [43]. These observations have led to the idea that in tumors, the cadherin-catenin complex is not only important for cell adhesion and migration, but probably also reflects on neoplastic cell growth and survival [43].

Stimulation with the pro-inflammatory cytokines, rHuIFN-γ and rHuTNF-α, did not alter the expression of E-cadherin and $CD44V_6$ in normal skin and BCC biopsies.

In order to evaluate the differences in expression between normal keratinocytes and BCC cells, an IEM study was undertaken. The results showed that E-cadherin and $CD44V_6$ were expressed in essentially all structures involved in cell-cell adhesion, such as desmosomes, adherens junctions and complex interdigitations, both on normal keratinocytes and on BCC cells. These findings indicate that the down-regulation of these molecules on BCC cells in vivo, observed at LM level, is not related to a specific absence or down-regulation of any specialized membrane structure and is most likely not caused by the presence of pro-inflammatory cytokines in situ.

Recently, the ultrastructural localization of CD44*standard* in normal epidermis has been reported by Tuhkanen et al [44]. In that study, its expression was most prominent in the spinous layers, which is in agreement with our results (**Table II, chapter 4**). In addition, in our study described in **chapter 4**, the expression of $CD44V_6$, instead of CD44*standard*, was investigated and a different IEM technique was used. We observed $CD44V_6$

expression in structures related to cell-cell adhesion, such as desmosomes and adherens junctions. Tuhkanen et al [44], however, observed expression of CD44*standard* predominantly on the plasma membrane facing the open intercellular spaces, but only minor expression in the desmosomes. These findings may indicate a different role for CD44*standard* and the for the different CD44 variant isoforms in cell-cell adhesion. Gebauer et al [45] reported that proteolytic enzymes degrade CD44 epitopes on malignant cells of mammary carcinoma and malignant melanoma cell lines. The enzymes reduced both the concentration and the function of these molecules on the malignant cells. Since the presence of proteolytic enzymes has been described in BCC [46,47], it is conceivable that they may be involved in the observed down-regulation of $CD44V_6$ on the BCC cells. Further investigations are necessary to clarify the exact role of down-regulation of E-cadherin, α-catenin and $CD44V_6$ in the different types of BCC.

Main conclusions and future directions

- Cytokeratin 8 is expressed in BCC and MAb 4.1.18 is very suitable to detect it. This MAb can be used to distinguish tumor cells from the peritumoral infiltrate. In future, this marker may be used to establish the exact origin of BCC in *in vivo* experiments.

- The absence or inconsistent expression of important molecules on the tumor cells, the presence of IL-10, the shedding of ICAM-1, and the down-regulation of IFN-γR expression may preclude an effective T cell-mediated immune response in BCC. Future experimental investigations into the immunology of BCC may involve the recently developed SCID mouse model for BCC [48]. In this *in vivo* model, BCC are grafted into healthy human skin previously transplanted on SCID mice. The major advantage of this model is that the take-rate of BCC is considerably improved and that the transplanted tumors preserve their morphological features. The *ex-vivo* studies previously described, as well as new therapeutic approaches may be validated using this model. Several possibilities are; 1) intralesional injection of IFN-γ; 2) prevention of shedding of ICAM-1; 3) strategies to inhibit IL-10, such as administration of Abs, to neutralize the immunosuppressive state in BCC; 4) localization of the cytokine-producing cell types present in BCC; 5) isolation and culturing of the autologous T cells and investigations into their state of activation and their ability to recognize and adhere to tumor cells after stimulation.

Investigations into these different aspects of BCC would eminently provide better insight into tumor immunology in general. For these purposes BCC is suitable because it is a common tumor and fresh samples are easily available.

- The adhesion molecules E-cadherin, α-catenin and $CD44V_6$, are down-regulated on BCC cells compared with the overlying epidermis. E-cadherin and $CD44V_6$ are expressed in all structures involved in cell-cell adhesion such as desmosomes, "adherens junctions" and "complex interdigitations". Ultrastructural investigations showed that the observed down-regulation in vivo is not related to a specific absence or down-regulation of any specialized membrane structure.

Concluding remarks

As a result of the attenuation of the stratospheric ozone layer, the amount of UVR reaching the earth is increasing, which has significant consequences for the human skin. In addition, the changes in recreational sun-exposure on holidays or on sunbeds may account for the increasing incidence of skin cancer, including BCC.

The investigations into BCC described in this thesis may provide a better understanding of the pathogenesis and pathophysiology of this tumor. Certain questions were answered, but new interesting questions have arisen. Therefore, it is imperative that more effort is undertaken for further research into the origin, development, immunological, and pathophysiological aspects of BCC and for developing new and more effective treatment modalities for these usually relatively innocuous, but sometimes devastating tumors.

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SAMENVATTING

Het basaalcelcarcinoom (BCC) is de meest voorkomende vorm van huidkanker van dit moment. Het is een maligne tumor, die zelden metastaseert, maar wel invasief groeit en daarbij kan leiden tot ernstige weefselbeschadiging. De belangrijkste risicofactor voor het ontstaan van deze tumor is blootstelling aan zonlicht in combinatie met een lichte huid (type 1 of 2).
Er bestaan meerdere histopathologische typen BCC met verschillende groei-eigenschappen. Er wordt nog steeds gespeculeerd over de cel van oorsprong van deze tumoren.
In **hoofdstuk 1** wordt een uitgebreide beschrijving gegeven van de belangrijkste eigenschappen van BCC, zoals de expressie van cytokeratinen en adhesiemoleculen en de immunologische aspecten. Tevens worden de doelstellingen van deze dissertatie beschreven.

Deze zijn kort samengevat:
1. Het vaststellen van het cytokeratine expressie patroon met het oog op het vinden van een geschikte histopathologische tumormarker.
2. Het verkrijgen van een beter inzicht in het ontbreken van een effectieve T cel-gemedieerde immuunrespons.
3. Het verkrijgen van een beter inzicht in het niet-metastaserende gedrag van BCC.

Hoofdstuk 2.1 beschrijft de resultaten van een immunohistochemish, lichtmicroscopisch, onderzoek waarin het cytokeratine (Ck) patroon van BCC wordt bekeken met behulp van een panel van monoklonale antilichamen (MAl). De expressie van Ck 8 werd waargenomen in alle 23 BCCs na kleuring met MAl 4.1.18. Twee andere MAls tegen Ck 8 toonden inconsistente aankleuring en kleuring met een vierde MAl was geheel negatief. Cks 7 en 19 werden inconsistent waargenomen en Cks 18 en 10 werden in geen van de BCCs gedetecteerd.
Hoofdstuk 2.2 beschrijft het onderzoek waarin de kleuringen met de vier MAls tegen Ck 8 werden vergeleken in licht- en electronen microscopie. De resultaten van dit onderzoek kwamen overeen met die van **hoofdstuk 2.1.**
We kunnen concluderen, dat Ck 8 tot expressie komt in BCC en dat MAl 4.1.18 het meest geschikt is om dit aan te tonen. Dit antilichaam kan tevens worden gebruikt om BCC cellen in coupes te onderscheiden van bijvoorbeeld infiltraatcellen.

In **Hoofdstuk 3** wordt ingegaan op het ontbreken van een effectieve T cel gemedieerde immuunrespons, ondanks de aanwezigheid van geactiveerde T cellen rondom de tumorvelden. Voor een effectieve T cel gemedieerde immuunrespons is een interactie tussen de T cel en de tumorcel noodzakelijk. De expressie van de verschillende moleculen op zowel de T cel als de tumorcel is daarbij van groot belang. De afwezigheid dan wel verminderde expressie van deze moleculen kunnen de oorzaak zijn van het ontbreken van een effectieve immuunrespons. Bepaalde cytokinen, zoals TNF-α en IFN-γ kunnen de expressie van deze moleculen op epitheelcellen induceren of verhogen. Immunosuppressieve cytokinen daarentegen, zoals IL-10, kunnen deze expressie juist verhinderen of afremmen.
In **hoofdstuk 3.1** wordt het effect van recombinant humaan (rHu)IFN-γ op biopten van BCC beschreven. Nadat de biopten 24 uur gekweekt waren met een relatief hoge dosis rHuIFN-γ werd op zowel de tumorcellen als de bovenliggende epidermis een significante toename van ICAM-1 expressie waargenomen. Tevens werd in het kweekmedium een significante toename van sICAM-1 gemeten. De opregulatie van ICAM-1 op de epidermale keratinocyten boven de tumorvelden was significant hoger dan die op de tumorcellen. Een "down-regulatie" van IFN-γ receptoren op de tumorvelden werd waargenomen. Dit zou een mogelijke verklaring kunnen zijn voor het verschil in ICAM-1 opregulatie.
In **hoofdstuk 3.2** wordt het effect van rHuIFN-γ op de expressie van MHC klasse I & II, CD40 en CD80 op de tumorcellen onderzocht. Tevens werd de hoeveelheid IL-10 bepaald in het kweekmedium. De expressie van ICAM-1 en CD40 was significant verhoogd op de epidermale keratinocyten na 48 uur kweken in de aanwezigheid van een hogere dosis rHuIFN-γ dan werd gebruikt in **hoofdstuk 3.1** Echter, slechts een trend werd waargenomen in de opregulatie van HLA-DR en CD80 expressie. Op de tumorcellen was alleen de ICAM-1 expressie significant verhoogd. CD40 expressie wasslechts matig verhoogd en de expressie van MHC klasse I, HLA-DR en CD80 op de tumorcellen bleef onveranderd. De hoeveelheid IL-10 in het kweekmedium van BCC biopten was significant groter dan in dat van de biopten van de normale huid. Dit geeft de aanwezigheid van IL-10 in BCC aan.
We kunnen concluderen, dat de afwezigheid van de essentiële moleculen, de verlaagde expressie van IFN-γ receptoren op BCC cellen, de "shedding" van ICAM-1 en de aanwezigheid van IL-10 mogelijke oorzaken kunnen zijn voor het ontbreken van een effectieve immuunrespons tegen BCC.

In **hoofdstuk 4** wordt een immunohistochemisch onderzoek beschreven, dat zich richt op de expressie van E-cadherine, α- en β-catenine en $CD44V_6$ (een vorm van CD44) in BCC

om een beter inzicht te verkrijgen in het feit dat BCC zelden metastaseert, maar wel degelijk invasief groeit. Op lichtmicroscopisch niveau werd een verlaagde expressie van E-cadherine, α-catenine en $CD44V_6$ op de tumorcellen gezien ten opzichte van de bovenliggende epidermis. De expressie van β-catenine was vergelijkbaar in de tumor en de epidermis. De invloed van eventueel aanwezige cytokinen op de verlaagde expressie van bovengenoemde moleculen werd bekeken met behulp van het kweeksysteem dat werd beschreven in **hoofdstuk 3**. Echter, IFN-γ en TNF-α hadden in dit systeem geen effect op de expressie van de adhesiemoleculen. Om de verschillen in de expressie tussen normale keratinocyten en tumorcellen te evalueren, werd met behulp van immunoelectronen microscopie gekeken naar de ultrastructurele lokalisatie van E-cadherine en $CD44V_6$. Expressie van deze moleculen werd waargenomen in alle structuren die betrokken zijn bij de cel-cel interactie, zoals desmosomen, "adherens junctions" en "complexe interdigitaties", zowel in keratinocyten als in de tumorcellen.
Derhalve kan worden geconcludeerd dat de zwakkere kleuring van E-cadherine en $CD44V_6$ in BCC op licht microscopisch niveau, niet wordt veroorzaakt door een specifieke afwezigheid of "down-regulatie" van bepaalde gespecialiseerde membraanstructuren. Tevens wordt het hoogstwaarschijnlijk niet veroorzaakt door de aanwezigheid van cytokinen.

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