Apoptosis and proliferation in basal cell carcinoma

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Apoptosis and Proliferation

in

Basal Cell Carcinoma

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Alles sal regt kom

In herinnering aan
Mama en Opa.

Voor Papa en Jan.

Aim of this thesis

In this thesis the growth characteristics of basal cell carcinoma (BCC), the most common malignancy among Caucasians, is studied using molecular markers. BCC is a non-melanoma type of skin cancer and accounts for approximately 80% of all skin malignancies and increasing incidence. The malignancy is characterized by a slow growth and rare metastases, but occasionally the tumor can infiltrate and cause severe damage to the underlying tissue. Since its constantly increasing incidence and morbidity it becomes more and more opportune to explore possible new non-invasive therapies based on basic knowledge of BCC.

Since in general tumor growth can be achieved by either deregulating the cell cycle, downregulation of cell loss, or both, this thesis concentrates on these processes of cell proliferation and programmed cell death (apoptosis).

Contents

11	Chapter 1 General introduction Molecular Cell Biology of Basal Cell Carcinoma
37	Chapter 2 A hairy issue Human follicular stem cells: Their presence in plucked hairs and follicular cell culture
51	Chapter 3 Bcl-2 family 3.1 Bax expression and growth behavior of Basal Cell Carcinomas
67	3.2 Expression of pro- and anti-apoptotic members of the Bcl-2 family and associated proteins in Normal Human Skin and Basal Cell Carcinoma
79	Chapter 4 Nuclear lamins in the skin Lamin expression in Normal Human Skin, Actinic Keratosis, Squamous Cell Carcinoma and Basal Cell Carcinoma
93	Chapter 5 Therapy of Basal Cell Carcinoma The garlic derived organosulfur component Ajoene decreases Basal Cell Carcinoma tumor size by inducing apoptosis
109	Summary
111	Samenvatting
113	Dankwoord
115	Curriculum Vitae
117	Publications

Chapter 1: **General introduction**

Molecular Cell Biology of Basal Cell Carcinoma

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Introduction

Basal cell carcinoma (BCC), a non-melanoma type of skin cancer, also called basalioma, basal cell epithelioma, rodent ulcer and Jacobs' ulcer, was first described in 1824¹ and is the most common cancer amongst Caucasians². BCC accounts for approximately 75% of all skin cancers. Mortality rates are relatively low³, but BCC may occasionally grow aggressively causing extensive tissue destruction⁴. Its ability to metastasize is low ($< 0.1\%$)⁵. Metastasis to lymph nodes, lung, bone and liver has been described^{6,7}.

BCCs can be subdivided according to their differences in histological appearance^{8,9}. The major histological patterns comprise nodular, micronodular, superficial and morpheaform BCC. The nodular type is characterized by a rounded mass of neoplastic cells with well-defined peripheral contours and peripheral palisading is well developed. The superficial type is defined by one or more tumor foci that extend from epidermis into the papillary dermis. Peripheral palisading occurs and the peripheral contours are smooth. The micronodular subtype is defined as small nodules and usually peripheral palisading is present. The morpheaform subtype demonstrates tumor islands of varying size with irregular outline and spiking configuration. Peripheral palisading is poorly developed. Mixed types of these histological patterns may occur, with the nodular-micronodular combination being the most common¹⁰. Furthermore, BCC with more nodular type in the center of the malignancy and morpheaform type at the periphery can occur. Finally, an adenoid pattern of BCC is seen in 1-7% of the tumors and is mainly associated with the nodular type BCC¹¹. Nodular (~60%) and superficial (~25%) BCCs are often considered as non-aggressive subtypes, whereas morpheaform (~2%) and micronodular (~15%) BCCs are often referred to as aggressive subtypes, and are associated with a higher risk of recurrence¹².

Risk factors

The risk for development of BCC is associated with several patient dependent, but also environmental factors.

- Environmental risk factors

BCCs generally occur on sun-exposed areas of the body¹³ and high-risk patients are often fair-skinned with a history of burning, not tanning, when exposed to strong sunlight¹⁴. Male gender, older age and number of previous second-degree sunburns are also factors indicating a higher risk for development of BCC¹⁵. Corona et al.¹⁶ showed in 2001 that there is a significant association between BCC development and recreational sun exposure during childhood and adolescence, as well as a strong relation with family history of skin cancer. Patients with a BCC located on the trunk are at increased risk of developing multiple BCCs, and these tumors develop at a faster rate than BCCs located elsewhere on the body¹⁷. Grossman et al.¹⁸ showed that there is a correlation between UV-B exposure and development of skin cancer. However, the significant number of BCCs arising on non-sun-exposed areas of the body

suggests that other risk factors may play a role in the development of BCC¹⁹. Diepgen et al.²⁰ found that chemical carcinogens such as arsenic, coal tar products and psoralens as well as ionizing radiation increase the risk of non-melanoma skin cancer. Combination of UV irradiation and arsenic exposure lead to an impaired process of nucleotide excision repair²¹. Excessive exposure to psoralen during UVA-treatment in psoriasis patients also results in an increased risk for BCC²². Prior non-diagnostic X-ray treatment for skin conditions also enhanced the risk for BCC¹⁹. Furthermore, several authors demonstrated an association between infection with the oncogenic types of human papilloma virus (HPV) and development of BCC²³⁻²⁵, while Harwood et al.²⁶ showed that HPV could abrogate UV-induced apoptosis. Furthermore, HPV DNA was detected in BCC patients by Barr et al.²³ and Weinstock et al.²⁵, suggesting HPV infection as a risk factor for developing BCC. With respect to the relation between smoking and skin cancer development, De Hertog et al.²⁷ showed an association with squamous cell carcinoma but not BCC, while Boyd et al.²⁸ recently proved that an association exists between smoking and BCC in young women. Furthermore, exposure to fiberglass dust and dry cleaning agents also enhance the risk for BCC development¹⁹.

- GST Polymorphism

Genes that mediate detoxification processes, including individual responses to UV irradiation by protecting from oxidative stress, are likely candidates for susceptibility genes for BCC²⁹. For example, glutathione S-transferase (GST) enzymes are part of the cells' defense mechanism against numerous harmful chemicals produced endogenously and in the environment³⁰. UV irradiation causes oxidative stress in the skin, which leads to lipid peroxidation and DNA hydroperoxide formation³¹. GST is responsible for the disposal of these potential mutagens³². Cytosolic GST activity in mammalian tissues is due to the presence of multiple GST isozymes, which can be assigned to five classes e.g. α , μ , π , σ and θ ³³. In human skin, GST activity can be found predominantly in sebaceous glands and in the outer root sheath of hair follicles, the π -class of GST being the predominant isozyme³⁴. GST- π has been suggested to be an oncofetal protein that is re-expressed during carcinogenesis³⁵. A significant increase in skin tumorigenesis is observed in mice lacking π -class GST³⁶. In humans, GST- π is mainly expressed in malignant melanomas³⁷, whereas BCCs show only a weak expression of the protein³⁸. Several polymorphisms in GST family members exist^{33,39} and have been associated with impaired detoxification, thus influencing the risk for several cancers, including non-melanoma skin cancer^{40,41}. A GSTT1 null genotype is associated with high UV sensitivity⁴², and GSTM1 null genotype also predisposes for BCC due to its role in defense against UV-induced oxidative stress^{29,43}. Polymorphism of GSTM3 was also shown to increase risk for BCC⁴⁴. Another genetic factor involved in detoxification of photosensitizing agents, and thus involved in BCC carcinogenesis is polymorphism of CYP2D6 (the gene encoding for cytochrome P450), which is correlated with increased number of BCC⁴³. Furthermore, CYP2D6 EM is associated with a multiple presentation phenotype of BCC^{45,46} and these patients are also at higher risk for developing BCC⁴⁷.

- Hereditary predisposition

In rare cases individuals can show an inherited predisposition for BCC. In 1973, Milstone and Helwig⁴⁸ noted that patients with Xeroderma Pigmentosum (XP), a group of rare autosomal recessive disorders characterized by severe photosensitivity due to various defects in the DNA repair of UV-induced lesions, are prone to develop cutaneous cancers, mostly squamous cell carcinoma (SCC) but also BCC⁴⁹⁻⁵¹. There are several variants of the disease, all caused by a different genetic defect in nucleotide excision repair (NER), global genome repair, transcription coupled repair or combinations thereof. Some of the genes involved are essential components of the TFIIH transcription complex; their absence is associated not only with UV sensitivity but also with sometimes severe neurological defects and growth retardation (as in de Sanctis-Cacchione)⁵². Interestingly, at least two types of XP are caused by defects in DNA helicases that are involved in NER and in transcription. Werner and Bloom syndromes are hereditary skin cancer disorders that are associated with helicase defects but, curiously, not with the development of basal cell carcinomas^{53,54}. Rothmund-Thomson syndrome, that in some cases is caused by defects in a DNA helicase, does predispose to BCC⁵⁵. The reason for this difference is poorly understood. The expression patterns of helicases may play a role but it is not clear why helicases should be tissue-specific. Chromosomal breakage disorders such as ataxia teleangiectasia and Nijmegen Breakage Syndrome do not predispose to BCC. Neither does Li-Fraumeni syndrome, which is caused by germline mutations in the p53 gene, or dyskeratosis congenita, a disorder associated with failure to maintain telomeres. Why these forms of genomic instability do not seem to be causally related to BCC is subject to speculation.

Moreover, patients with the nevoid BCC syndrome (NBCCS) or Gorlin syndrome⁵⁶, also show a rapid development of numerous tumors at young age. Whereas XP is an autosomal recessive disorder, NBCCS is an autosomal dominant disorder⁵⁷. A human homologue to the *Drosophila* segment polarity gene *patched*, *PTCH* is mutated in NBCCS patients, suggesting a contribution to the tumorigenesis^{58,59}. Since NBCCS patients normally inherit one mutated copy of the *PTCH* gene, tumors are likely to arise after inactivation of the remaining allele⁶⁰. Haplo-insufficiency of the *PTCH* gene is probably responsible for the dysmorphisms. XP patients and sporadic BCCs may also show mutations in the *PTCH* gene⁶¹⁻⁶³. The *PTCH* gene product is involved in the so-called Sonic Hedgehog-signaling pathway, which is involved in embryonic development⁶⁴. Sonic hedgehog (*shh*) is expressed in the Hensen node, the floor plate of the neural tube, the early gut endoderm, the posterior limb buds and throughout the notochord, and encodes a signal responsible for patterning the early embryo⁶⁵⁻⁶⁷. At the cell surface, *patched* and *smoothed* form a receptor complex for sonic hedgehog, which is a secreted molecule that influences the differentiation of a variety of tissues such as brain, spinal cord, limbs and axial skeleton⁶⁸. When sonic hedgehog binds to *patched*, it releases *smoothed*, a transmembrane signaling protein, which is inhibited by binding to *patched* protein⁶⁹. High frequency of mutations in *smoothed* and *patched* in BCCs, resulting in continuous activation of target genes, indicates that a disturbed hedgehog pathway, resulting in excessive signaling, may be an important carcinogenic route⁷⁰. UV irradiation enhances BCC development in

PTCH mutated mice⁷¹. Of the sporadic BCCs 20% show smoothened mutations⁷² and 30-40% patched mutations⁶². In XP patients, the majority (~80%) of PTCH mutations are UV-induced⁷³, more frequently than in sporadic BCCs⁶², where UV-signature mutations are seen in less than 50%⁷⁴. PTCH2, which is 57% identical to PTCH1, also serves as a receptor for hedgehog and related factors⁷⁵. Mutations occur in sporadic BCC⁷⁶, and it has been shown that when PTCH1 is mutated, PTCH2 mRNA is upregulated⁷⁵.

Finally, Rombo and Bazex syndromes are known to predispose to BCC. Both are characterized by the presence of numerous small cysts in the face chest, as well as by hypotrichosis. The cysts contain vellus hairs^{77,78}. Rombo syndrome is distinguished by striking degeneration of elastic fibrils in sunlight-exposed areas causing dramatic skin alterations called atrophoderma vermiculatum^{77,78}. In Bazex syndrome, so-called ice-pick scars are seen on the backs of the hands. This disorder is X-linked and has been mapped to Xq24-27. Rombo syndrome is probably autosomal dominant, but elsewhere very similar to Bazex.

- Genetic changes

The most common genetic aberrations in human skin cancers are found at the level of the p53 gene, which mediates cell cycle arrest after DNA damage⁷⁹. The p53 gene encodes a phospho-protein that is involved in cell cycle control⁸⁰. In response to cellular stress, for example DNA damage, p53 is activated through phosphorylation^{81,82}. Mdm2 can associate with p53 and regulates its level and activity depending on the phosphorylation status of p53. When dephosphorylated, p53 can bind to mdm2 and is then being degraded through the ubiquitin-proteasome pathway^{83,84}. In response to DNA damage, p53 is phosphorylated and becomes detached from mdm2, resulting in stabilization and activation of target genes regulated by p53⁸⁵. The response to DNA damage is either growth arrest, senescence or apoptosis⁸⁶. The relative cellular content of p53 determines the response following DNA damage; when the content is low to moderate, cells will go into cell cycle arrest to allow DNA repair, but when p53 levels are high, cells will progress to apoptosis⁸⁷. P53 is capable of stimulating pro-apoptotic Bax expression^{88,89} (see also below). In normal skin, wild type (WT) p53 is not detectable but appears within 2 hours after UV irradiation, with peak levels at 24 hours after irradiation and again undetectable levels at 36 hours after irradiation⁹⁰. Mutant p53 can accumulate in cells and p53 mutations have been detected in about half of all BCCs^{91,92}. It was furthermore proven that histologically proven aggressive BCCs are significantly associated with increased p53 expression, most probably representing the mutated form. It is, however, strikingly that patients suffering from Li-Fraumeni syndrome do not show increased incidence of BCC. In BCC patients, a study comparing sunscreen users and non-users, it was shown that sunscreen users showed a significantly lower level of p53 mutations in their BCCs than non-users⁹³. Mutation-hotspots have been identified, with two-third of the mutations occurring at nine different sites⁹⁴. Inactivation of p53 occurs predominantly by point mutation of one allele followed by loss of the remaining wild type allele⁹⁵. The p53 gene shows UV-signature mutations i.e. predominantly C(C) -> T(T) conversions^{94,96}. In 33% of Korean BCC patients p53 mutations were detected⁶¹ and up to 50% of the Caucasian BCC patients showed this mutation^{91,92}, thus suggesting that different ethnic factors play a role in BCC carcinogenesis.

Although at the moment no explanation at the molecular level is available for this finding, polymorphisms in genes may be related to this phenomenon, as for example the MC1R gene, coding for the receptor of melanocortin 1 and involved in the regulation of pigmentation⁹⁷. Polymorphism in this gene is associated with squamous cell carcinoma or melanoma in the Irish and Scottish population^{98,99}.

P63, a p53 homologue, encodes multiple products and is restricted to cells with high proliferate potential and absent from cells undergoing terminal differentiation¹⁰⁰. P63 has a nucleoplasmic distribution pattern in the basal compartment of stratified epithelia such as skin, tonsil, bladder and certain subpopulations of basal cells in prostate, breast, uterine cervix and bronchi¹⁰¹⁻¹⁰³. P63 deficient mice have striking developmental defects such as absence or truncation of limbs, absence of hair follicles, teeth and mammary glands, and the skin lacks stratification and differentiation¹⁰⁴. This indicates that p63 is essential for several aspects of differentiation during embryogenesis. Several isoforms of p63 can bind to p53 consensus-sequences and activate p53 target-genes. Isoform TAp63 γ is capable of inducing cell cycle arrest and apoptosis¹⁰⁵. The ΔN isoforms, lacking the N-terminus, are unable to induce transcription, and have an anti-apoptotic effect by rendering p53 and TA isoforms inactive. P63 is only rarely mutated¹⁰⁶. It was furthermore shown that p63 functions not only as a stem cell marker of keratinocytes¹⁰⁷ but it also suggested that it maintains the stem cell phenotype¹⁰⁸. In consistency with its basal cell localization in normal epidermis, BCC cells express p63^{103,109}. It was shown, that aberrant expression of p63 altered the UVB-induced apoptotic pathway suggesting that downregulation of this protein in response to UV irradiation is important in epidermal apoptosis¹¹⁰.

- Immunosuppression

Organ transplant recipients are considered to be at greater risk for development of malignancies because of the prolonged, often life long, immunosuppression therapy¹¹¹⁻¹¹³. Skin cancer is the most common malignancy occurring in the setting of solid-organ transplantation and immunosuppression, and its incidence increases substantially with extended survival after transplantation¹¹⁴. Surprisingly, squamous cell carcinoma (SCC) occurs more frequently in transplant patients¹¹⁵, whereas in the general population BCC occurs 3-6 times more frequent than SCC¹¹⁶. It was shown in Australian heart transplant recipients that the number of skin cancers is significantly correlated with both age at transplantation and duration of follow-up¹¹⁷. In Europe, 40% of the renal transplant recipients develop skin cancer within 20 years after grafting¹¹⁸. Heart transplant recipients are at higher risk than kidney transplant recipients most probably due to the fact that they receive higher doses of immunosuppression agents¹¹⁹, but it cannot be excluded that the different types of immunosuppressive agents have different effects in this respect.

People suffering from AIDS also have shown an elevated risk for the development of BCC¹²⁰ and HIV patients with BCC show more frequently blue eyes, blond hair, family history and extensive prior sun exposure¹²¹.

The major histocompatibility complex (MHC) genes code for membrane proteins that play an important role in controlling immune responses¹²². There are two classes of genes, Class I (HLA-A, -B, -C) and Class II (HLA-DR, -DQ, DP),

which play a role in host defense against development and spread of tumors¹²³. For example, loss of Class I antigens is related to tumor progression in melanomas¹²⁴. Furthermore, abnormalities in cell-mediated immunity have been reported in patients with multiple BCCs¹²⁵. Whereas normal skin shows high levels of Class I molecules, BCC shows either complete absence or heterogeneous expression¹²⁶. All Class I negative tumors were histologically proven aggressive, whereas all non-aggressive BCCs were class I positive. The low levels or absence of expression of Class I antigens may result in escape from recognition by cytotoxic T cells, which then facilitates tumor growth¹²⁷. Evidence for the involvement of HLA-genes in the development of skin cancer was provided by Bouwes Bavinck et al.¹²⁸ These authors showed that the presence of HLA-DR7 and a decrease of HLA-DR4 are significantly associated with BCC. This corroborates the previous finding of Rompel et al.¹²⁹ that HLA-DR4 is decreased in BCC, especially in patients with multiple BCCs located on the trunk¹³⁰. The authors suggested a protective role for HLA-DR4 against development of BCC. HLA-DR1 is weakly associated with the development of multiple BCCs at an early age¹³¹. Furthermore, Bouwes Bavinck et al. presented two studies showing a correlation between HLA-A11 expression and skin cancer in immunosuppressed renal transplant recipients^{132,133}. One of these studies showed that HLA-A11 was associated with resistance to skin cancer in renal transplant recipients¹³², while another study, in Australia, showed that renal transplant recipients with HLA-A11 had an increased risk for developing skin cancer¹³³. This apparent discrepancy may be the result of different genetic background and differential environmental factors.

Although human papilloma viruses (HPV) has been associated strongly with malignant progression of warts to SCC and with epidermodysplasia verruciformis¹³⁴, different oncogenic subtypes of the virus were found in 60% of BCCs from immunosuppressed patients in contrast to 36% of BCCs from non-immunosuppressed patients, suggesting that these viruses may be involved in the development of BCC¹³⁵. In renal transplant recipients with skin cancer HPV 5/8 DNA could be detected²³, and Weinstock et al.²⁵ suggested immunosuppression to be a factor in BCC carcinogenesis by affecting HPV infection.

Precursor cells

Several cell types have been suggested to be the precursor cells or stem cells for BCC, i.e. interfollicular basal cells, basal keratinocytes from hair follicles or sebaceous gland cells¹³⁶⁻¹³⁸. In general stem cells have a relatively undifferentiated and slow cycling phenotype, but can be stimulated to proliferate and give rise to transient amplifying cells which have a limited proliferate potential¹³⁹. Stem cells may be the target of carcinogens and as such play an important role in tumorigenesis. For example, one observation that suggests that stem cells are the most likely cells of origin for human skin cancer, is the fact that sunlight exposure in childhood may contribute to tumors many decades later¹⁴⁰. As suggested by Taylor et al.¹⁴¹ the ultimate source of stem cells in the skin is the hair follicle. These stem cells are most likely located in the bulge

region of the outer root sheath (ORS)¹⁴². As a result, hair follicles play a potentially important role in skin homeostasis, wound healing and tumorigenesis¹³⁹. Chemically induced BCCs in rats arise from hair follicles¹⁴³, while in humans, BCCs can apparently arise from both follicular and interfollicular epidermis¹³⁶. Histologically, BCCs may resemble hair follicles¹¹, and may show characteristics from both bulge region stem cells and transient amplifying cells¹⁴⁴. In particular, a small fraction of BCC may histologically resemble trichoepithelioma, a benign hair follicle tumor¹⁴⁵. Especially the suprabullar region of the ORS of the hair follicle shows an almost identical immunohistochemical profile^{137,146}, suggesting a role for the lower ORS in the generation of BCC. The hair follicle hypothesis is furthermore supported by the fact that when a carcinogen is added in anagen-phase, in which the hair follicle bulge region cells undergo transient amplification, BCCs are generated more frequently¹⁴⁷. Furthermore, BCCs seldomly occur on non-hairy skin¹⁴⁴. Support for the hair follicle hypothesis can be found in the expression of the basal cell adhesion molecule B-CAM in normal and diseased skin¹⁴⁸. The fact that this cell surface protein is preferably expressed in suprabasal cell layers and the outer root sheath of the hair follicle, and also shows high levels of expression in BCCs, suggests BCC originating from hair follicles rather than from basal keratinocytes, which are negative for B-CAM in normal skin. BCC However, based on the lack of cytokeratin (CK) 15 expression in the tumor cells, BCCs do not differentiate towards hair bulge cells¹⁴⁹. In support of the interfollicular origin of BCC Howell et al.¹⁵⁰ reported the tiny pits in the epidermis of palms and soles characteristic for Gorlin syndrome⁵⁶ (also called nevoid basal cell carcinoma syndrome) occasionally show basaloid budding into the dermis, and have therefore been suggested to resemble tiny BCCs.

Carcinogenesis

Tumor formation results from a disruption of the normal balance between cell proliferation and cell death¹⁵¹. Three large categories of genes affect cellular proliferation and survival, i.e. growth promoting oncogenes, tumor suppressor genes and mutator or caretaker genes¹⁵². The normal counterparts of oncogenes, i.e. the proto-oncogenes, are crucial in regulating normal cell cycling and division, differentiation and apoptosis¹⁵³⁻¹⁵⁵. When these become mutated or amplified they can overcome the normal restraints of cell growth^{156,157}. Tumor suppressor genes negatively regulate cell growth or promote cell death, and mutations or deletions of such genes have been reported in various types of cancer¹⁵⁸⁻¹⁶⁰. Activation of proto-oncogenes and inactivation of tumor-suppressor genes leads to an accumulation of genetic abnormalities¹⁶¹. Defects in tumor suppressor genes appear to be more common than defects in oncogenes¹⁶². Mutator or caretaker genes maintain the genome integrity and when their function is altered, mutations can accumulate more frequently¹⁵².

- Proliferation versus differentiation in BCC

Proliferation indices vary greatly for the different subtypes of BCC, but in general relatively high percentages of proliferating tumor cells are found¹⁶³⁻¹⁶⁶. Based on immunohistochemical detection of the proliferation marker Ki-67 on average 20% of the cells in BCC are proliferating^{164,165,167,168}. The proliferating cell nuclear antigen (PCNA) is present in <10% of non-recurrent BCCs, while recurrent BCCs show PCNA expression in >30% of the tumor cells^{169,170}. In nodular and superficial BCC the proliferative activity is mainly restricted to the periphery of the tumor nests, whereas morphea-like tumors show a more scattered pattern of proliferating cells^{164,168}. An explanation for the zonal distribution of proliferation-potent cells may be that tumor cells migrating towards the center of the tumor nests become more differentiated, or have less access to nutrients, resulting in lower proliferation potential¹⁷¹.

Markers for arrest of cell proliferation include the A-type lamins, also designated Statins¹⁷². Nuclear lamins are intermediate filament proteins that form a network at the nucleoplasmic site of the nuclear membrane and can be divided in two sub-types, i.e. A-type lamins (lamin A, lamin A Δ 10 and lamin C) and B-type lamins (lamin B1 and lamin B2)^{173,174}. Aberrant expression patterns of lamins have been described in cancer and it is thought that the nuclear matrix plays a role in carcinogenesis¹⁷⁵. In general, A-type lamin expression is correlated with a non-proliferating, differentiated state of cells and tissues¹⁷⁶, and therefore altered expression of A-type lamins can be expected in cancer. In BCC, it was recently reported by Venables et al.¹⁷⁷, that the absence of lamin A correlated with rapid growth, while the absence of lamin C correlated with slow growth of this skin tumor. It was furthermore reported, that the expression of A- and B-type lamins varies with differentiation in normal epidermis¹⁷⁸ and skin tumors^{178,179}. These authors support the idea that expression of A-type lamins, but not B-type lamins is associated with the differentiation phenotype of the tumors. Recently, Tilli et al.¹⁸⁰ reported four stages in BCC development based on different patterns of A-type lamin expression. Stage 1 comprises lamin A negative, Ki-67 positive BCCs, representing the origin of BCC, while stage 2 comprises lamin A positive, Ki-67 positive BCCs. As tumor growth slows down, lamin C is first re-located to the nucleolus in stage 3 and in stage 4 lamin C expression is largely diminished.

The fact that BCC show relatively high percentages of proliferating tumor cells is not in line with the clinical finding that BCC is in general slow-growing¹⁸¹. Therefore, the process of cell loss must be considered as an important factor in the net growth of BCC. Already in 1972, Kerr¹⁸² reported that a high apoptotic rate in BCC might account for the paradoxically slow growth rate. Furthermore, Mooney¹⁸³ showed that indeed BCCs exhibit a high apoptotic rate as based on in situ end labeling.

- Apoptosis in epidermis and BCC

Apoptosis, a form of programmed cell death, is characterized by cell shrinkage and fragmentation¹⁸⁴. Apoptosis is amongst others necessary for the correct development of an embryo¹⁸⁵, and to eliminate auto-reactive lymphocytes¹⁸⁶. Abnormal, unwanted or damaged cells are removed by apoptosis without the involvement of the immune system, but through rapid phagocytosis of apoptotic cells before lysis, which prevents inflammation¹⁸⁷. In this respect the process of

programmed cell death can be clearly distinguished from accidental cell death, i.e. necrosis.

During the different phases of apoptosis various sets of molecules act in an orchestrated fashion. These include:

1. Death ligands and death receptors. The family of death receptors is characterized by two to five copies of cysteine-rich extracellular repeats and a death domain within the intracellular carboxy-terminus of the receptor (the death domain)¹⁸⁸. When these death receptors are bound by ligand, apoptosis can be induced. Fas is an example of a type I transmembrane receptor which mediates apoptosis upon binding of the oligomerizing Fas Ligand (FasL)¹⁸⁹. Fas is expressed on several different cell types, while expression of FasL is restricted to immune cells, including T and B lymphocytes, macrophages, and natural killer cells^{190,191}. Ligation of FasL to Fas causes rapid death-inducing signaling complex formation, which recruits and activates pro-caspase-8, thus triggering the apoptotic caspase cascade (see below).

2. Bcl-2 protein family. Many studies concentrate on the Bcl-2 family of apoptosis-regulating proteins^{192,193}. Bcl-2 was first discovered in B-cell lymphomas showing a t(14:18) translocation¹⁹⁴, resulting in a Bcl-2-Immunoglobulin-heavy chain fusion-gene¹⁹⁵. This leads to the over-expression of the anti-apoptotic Bcl-2 protein. The protein has been shown to suppress apoptosis induced by various stimuli, such as depletion of IL-3 and IL-4^{196,197}, p53-induced apoptosis¹⁹⁸, glucocorticoid treatment¹⁹⁹, and c-myc induced apoptosis²⁰⁰. Bcl-2 expression has been localized to long-lived (stem) cells in self-renewing human tissues¹⁵³. The protein is associated with the membranes of mitochondria, endoplasmic reticulum and nucleus²⁰¹, and bears 7 phosphorylation sites of which ser70 is critical for the apoptosis suppressing function of Bcl-2²⁰².

A large number of Bcl-2 related proteins^{192,193,203,204} have been isolated, which can act either as apoptosis-inducing (e.g. Bax, Bcl-xs) or apoptosis-suppressing agents (Bcl-xl). Heterodimerization between these family members determines whether a cell will die or not to die^{205,206}.

3. Caspases. The aspartate-specific cysteine protease (caspase) cascade appears to be the main pathway for clearance of cellular constituents during the execution phase of apoptosis²⁰⁷. Several human caspases have been identified that share similarities in amino acid sequence, structure and substrate specificity^{208,209}. Caspases show a high specificity for the conserved QACXG sequence, resulting in the cleavage after aspartic acid (Asp) residues²¹⁰. The caspase family comprises apoptotic initiators (e.g. caspase-2, -8, -9 and -10) and apoptotic executioners (e.g. caspase-3, -6 and -7)²⁰⁸. Caspase-3 seems to be responsible for the majority of apoptotic effects, and is supported by caspase-6 and -7. These three executioner caspases are important in the cleavage and degradation of several substrates, target-proteins that are involved in RNA splicing, DNA repair, and scaffolding of the cytosol and the nucleus. Upon induction of apoptosis, caspase-3 cleaves the inhibitor of the caspase-activated DNase, resulting in degradation of DNA into oligonucleosomal fragments^{211,212}. Lamin A is cleaved by caspase-6^{213,214}, while also cytoskeletal filaments such as cytokeratins are cleaved by caspase-6²¹⁵.

The externalization of phosphatidyl-serine at the cell membrane during apoptosis is also caspase-dependent²¹⁶. The nuclear matrix protein poly(ADP-ribose) polymerase (PARP) is also proteolysed by caspases during apoptosis²¹⁷.

Inhibitors of apoptosis proteins (IAPs), which are constitutively present in cells^{218,219}, for example bind to and inhibit caspase-3 and -7 as well as procaspase-9, but not caspase-1, -6, -8 or -10.

When apoptosis occurs inappropriately it may cause degeneration of normal tissue architecture or function. On the other hand, when apoptosis fails to occur this can give rise to deregulation of tissue homeostasis, as a result of which neoplasms can arise.

Apoptosis in the epidermis is a common phenomenon. In the morphogenesis of human fetal skin and maintenance of adult epidermis apoptosis plays a pivotal role²²⁰. For example, the apoptosis machinery is activated during the normal terminal differentiation process in keratinocytes²²¹. In fetal skin, cells undergoing apoptosis are present in several epidermal cell layers, whereas in neonatal epidermis these are found in the terminally differentiating granular cell layer, and in adult skin also the spinous cells show occasional apoptosis²²⁰. Furthermore, apoptosis occurs upon excessive UV light exposure, resulting in un-repairable DNA damage²²². A significantly negative correlation between the expression of either p53 or Bcl-2 with the development of BCC has been described previously²²³. Mutation of p53 or over-expression of Bcl-2 is sufficient to enhance formation of BCC by suppressing apoptosis^{224,225}.

Altered expression of Bcl-2 family member proteins in non-melanoma skin cancer has been reported extensively before²²⁶, suggesting that deregulation of expression of these proteins may be a possible explanation of the indolent growth-behavior of BCC^{168,227}. Bcl-2 is in general homogeneously expressed in BCC^{168,228-230}, while also the apoptosis-inducing Bax protein is expressed at high levels^{168,226}. These data clearly show that a considerable proportion of cells in BCC are in principle capable of undergoing apoptosis, corroborating the earlier finding of Mooney¹⁸³.

Another apoptosis inhibitor protein called survivin is expressed in 81% of BCC, whereas it is not detected in normal skin, suggesting a contribution to the progression of BCC²³¹.

Also Fas-mediated apoptosis may be important for skin homeostasis. Hill et al.²³² suggested that deregulation of Fas-FasL interactions may be central to the development of skin cancer. In normal skin, Fas is expressed in cytoplasmic membranes of the basal cell layer, while after sun exposure the expression of Fas is upregulated in the entire epidermis. After further UV exposure, Fas expression is again down regulated, resulting in a negative staining in BCCs²³³. BCCs express however strongly and diffusely FasL, providing evidence for an escape from local immune surveillance by the induction of apoptosis in the peripheral T lymphocytes²³⁴.

Induction of apoptosis in therapy of BCC

Different surgical and non-surgical therapies are available for the treatment of BCC²³⁵. Medical history of the patient, age, tumor localization and size, physical condition, histological outcomes and cosmetic aspects will eventually determine the choice of therapy. Furthermore, Telfer et al.²³⁶ published guidelines for the management of BCC, presenting evidence-based guidance for treatment. In spite of the fact that surgical excision is still the most prominent therapy used, non-invasive therapies like photodynamic therapy (PDT)²³⁷ or topical application of 5-fluorouracil²³⁸ are currently becoming more and more interesting in selective cases, especially because of the improved cosmetic outcome.

Many currently used anti-neoplastic agents exert their therapeutic effects through the induction of apoptosis. Different cell types vary profoundly in their susceptibility, suggesting the existence of distinct cellular thresholds for apoptosis induction²³⁹. For example, BCC cells over-expressing IL-6 are resistant to UV irradiation and PDT-induced apoptosis²⁴⁰. Furthermore, it was shown that de novo p53 synthesis or stabilization of p53 is essential to induce apoptosis in BCC²⁴¹. Over-expression of the anti-apoptotic Bcl-2 has also been linked to resistance of cancers to various chemotherapeutic drugs²⁴².

In BCC Interferon (IFN)- α induces apoptosis and is thus effective in the treatment²⁴³. Untreated BCC cells express FasL but not the receptor, but in IFN-alpha-treated BCC patients the tumor cells express both FasL and receptor, whereas the peritumoral infiltrate mainly consists of Fas-receptor-positive cells²⁴⁴. Upon IFN- α treatment the BCCs therefore most likely regress through apoptosis.

Topical treatment of BCC with 5-fluorouracil (5-FU) has also been proven to be very successful. Up to 90% of treated BCCs show regression when 5-FU is applied in a phosphatidyl choline-based cream²⁴⁵ or when it is locally injected in an epinephrine containing gel²³⁸. The regression of the tumors treated with 5-FU is probably caused by enhancing apoptosis in the tumor cells²⁴⁶.

Recently, Nakaseko et al.²⁴⁷ reported that apoptosis is involved in regression of the lesion after photodynamic therapy (PDT) in Actinic Keratosis. This therapy is also used for treatment of BCC²⁴⁸, where tumor cells may also undergo apoptosis.

Retinoids (vitamin A metabolites and analogs) have also been shown to have suppressive effects on tumor promotion, and the mechanism appears to be associated with modulation of growth, differentiation and apoptosis²⁴⁹.

Also more and more phytochemicals are being applied in cancer prevention and therapy²⁵⁰. Recently, Levin and Maibach²⁵¹ published an overview of alternative drugs and treatments in dermatology. It was shown that by application of green tea polyphenolic fractions reduced the UV-induced erythema, gave rise to a decrease in sunburns and could also reduce UV-induced mutations in DNA²⁵². Oral and topical application of black tea extracts also decreased photochemical damage to the skin²⁵³. Furthermore, in mice bearing skin tumors, tumor growth was inhibited by 70% after treatment with black tea, which was established by inhibition of proliferation and enhanced apoptosis²⁵⁴. Ajoene, an organosulfur compound of garlic²⁵⁵, has been shown to induce apoptosis in human promyeloleukemic cells²⁵⁶. Recently, it was shown that ajoene can induce

apoptosis in the human keratinocyte cell line HaCat and has a diminishing effect on BCC in vivo by downregulating the expression of the apoptosis-suppressing protein Bcl-2 (Tilli in press).

Based on the foregoing it is our firm believe that the development of future therapies for BCC will be strongly influenced by attempts to specifically induce apoptosis in these tumor cells.

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Chapter 2: A hairy issue

Human follicular stem cells: Their presence in plucked hair and follicular cell culture

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Summary

Background / Objectives

A considerable portion of the hair follicle remains attached to plucked hair and can be used for follicle cell culture. In this study we have phenotyped these cells in an attempt to identify the stem cell fraction. Reports in the literature have indicated that this cell population may be positive for cytokeratin (CK) 19. Since stem cells in general need to be protected from apoptosis, the presence of the apoptosis suppressing Bcl-2 protein, together with the absence of the apoptosis promoting Bax and the CK profile may, be used as an indicator of the stem cell population in the hair follicle, and in cultures of hair follicle cells.

Methods

Hair follicles from skin biopsies and plucked hair were derived from the scalps of healthy volunteers. Follicular cells were cultured from the plucked hairs. These hair follicles, plucked hairs, and cultured cells were examined for their CK profiles, which are indicative of the type of cell (basal/stem cells), and for their status with respect to the proliferation marker Ki-67, Bax, and Bcl-2.

Results

We found co-expression for CK19 and Bcl-2, but not Bax in two distinct areas, localized in the upper and lower third of the follicle from both skin biopsies and plucked hairs, while proliferation markers were negative in these areas. CK19 and Bcl-2 were also co-expressed in combination in a fraction of the follicular cell culture. The skin basal cell marker CK14 could be found throughout the outer root sheath of the hair follicle from both skin biopsies and plucked hairs, as well as in the follicular cell culture.

Conclusions

Thus, CK19 / Bcl-2-positive and Bax-negative cells can be obtained from cells derived from plucked hair and are retained in cultures made from these cells. If this phenotype represents follicular stem cells, our finding endorses the assumption that stem cells are located in the bulge area of the hair follicle, as we did not find them in or near the dermal papilla.

Introduction

Although a considerable portion of the hair follicle is attached to plucked hair, transplantation of a plucked hair does not result in normal hair growth¹, in contrast to hair from micro-punch grafts². This can be explained by the fact that the structures of the hair follicle that are retained in the skin after the hair is plucked, are necessary for normal hair growth. However, when outer root sheath (ORS) cells derived from plucked hairs are cultured, they can develop into a differentiated epidermis³, suitable for use in skin grafts⁴. We therefore speculated that the plucked hairs themselves might contain epidermal stem cells.

When plucked hair is dissected, the various transverse sections have been shown to exhibit different proliferative and differentiative characteristics⁵. It has been suggested that adult human follicular stem cells from terminal hairs are situated in the bulge area of the follicle. These cells have been suggested to exhibit various specific biochemical properties. For example they have epidermal growth factor (EGF) receptors, show alpha2beta1- and alpha3beta1-integrin expression⁶, high levels of alpha6-integrins and low levels of the proliferation marker 10G7⁷, and stain positively for platelet-derived growth factor (PDGF)-A/PDGF-B ligand chains. They do not contain nectadrin, or heat-stable antigen (CD24), a glycoprotein thought to be involved in cell-cell adhesion and signaling, which is also expressed in the outer epithelial sheath of human hair follicles and in glabrous epidermis^{8,9}. However the CK apoptosis resistance profiles seem to have become key indicators of a stem cell phenotype.

During development into a terminal hair, the CK profile of the follicular keratinocyte changes. It has been proposed that CK19 is an indicator of the stem cell population¹⁰⁻¹³. CK19 is present in immature epithelial progenitor cells¹⁴, but in the hair follicle it is specific for follicular stem cells^{10,11}. In adult hair follicles, CK19 can be found in the outermost cells of the ORS at the isthmus and in some cells of the lower ORS¹⁰. It is proposed that the actual follicular stem cells are CK19-positive and lack connexin (Cx) 43, a specific differentiation marker for a gap junction protein.

A balance between cell proliferation, differentiation and apoptosis is essential for hair growth^{15,16}, while stem cells must be protected against apoptosis. This protection is achieved by proteins such as Bcl-2, while Bax, a conserved homologue that heterodimerizes with Bcl-2 promotes cell death¹⁷. The characteristics of the CK profile, in particular the expression of CK19, but also CK5 and CK14 as basal cell markers¹¹, together with the expression of Bcl-2 and absence of Bax expression, may therefore be used as indicators of the stem cells in the hair follicle.

The aim of this study was to localize the follicular stem cells in the hair follicle from skin biopsies and to characterize them on the basis of their CK phenotype, presence of Bcl-2, and absence of Bax. Furthermore, we wished to determine whether these cells are present in plucked hair and preserved in cell cultures derived therefrom.

Materials and Methods

Skin biopsies

Five healthy volunteers, three males and two females between the ages of 28 and 53 years (mean age 37 years) donated skin biopsies.

Biopsies

Four 3-mm punch biopsies were obtained from the occipital area of the scalp after local anaesthesia with lidocaine 2%. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Samples were then cut into 5- μm thick sections in a vertical direction and carefully placed on Superfrost plus slides (Menzel-Glaser, Germany).

Plucked hair

Plucked hairs were obtained by removing the hairs with a depilation forceps from the occipital area of the scalp. Hair follicles in the anagen phase were selected under a dissection microscope, and embedded in Tissue Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands), and directly cut into 5- μm sections as described above.

Cell cultures

Plucked hairs were placed in a Petridish with Defined Serum-Free Keratinocyte Growth Medium (dSFk; Life Technologies B.V. Breda, The Netherlands). The nonviable, keratinised part of the hair follicle was removed under a dissection microscope. The hair follicles were subsequently put in a sterile culture disk and incubated in dSFk containing 20U/ml dispase (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 30 minutes in a CO_2 incubator at 37°C . After this pre-incubation step, the hair follicles were transferred to a 24-well culture disk containing dSFk with 500 $\mu\text{g/ml}$ penicillin (Life Technologies B.V. Breda, The Netherlands) and 0.25 $\mu\text{g/ml}$ streptomycin (Life Technologies B.V. Breda, The Netherlands), and placed for 14 days at 31°C in a humidified atmosphere containing 5% CO_2 . The culture medium was carefully removed every three days and replaced by fresh culture medium. The cells remained attached to the hair follicles during this culture period. After 14 days the culture medium was removed and replaced by a 0.5mg/ml trypsin, 0.2mg/ml EDTA (ethylene diaminetetraacetic acid) solution (Life Technologies B.V. Breda, The Netherlands), and incubated for 5 minutes at 37°C in this medium. After this incubation period clusters of cells were released from the hair follicles. These were harvested by centrifugation at 300 g at 4°C for 5 minutes in an Eppendorf 5804R Centrifuge (VWR International, The Netherlands). Subsequently the trypsin/EDTA medium was removed and replaced by culture medium. Cytospins were made from these cells in the Cytospin 3 (SHANDON, Zeist, The Netherlands) by centrifugation at 600 rpm for 5 minutes.

Table 1: Antibodies and their dilutions used in this study

Antibody	Isotype	Antigen	Dilution	Source
Basal cell markers				
LL002	mouse IgG ₃	CK 14	UD	Purkis et al. ³⁹
RCK107	mouse IgG ₁	CK 14	UD	Wetzels et al. ⁴⁰
RCK108	mouse IgG ₁	CK 19	1:5	Kwaspen et al. ⁴¹
LP2K	mouse IgG _{2b}	CK19	UD	Stasiak et al. ⁴²
Proliferation markers				
LL025	mouse IgG ₁	CK 16	1:10	Wetzels et al. ⁴⁰
MIB-1	mouse IgG ₁	Ki-67	1:25	Immuno Technology, Marseille, France DAKOA/S,Glostrup, Denmark
A0047	rabbit IgG	Ki-67	1:100	
Apoptosis markers				
N-19	rabbit IgG	Bcl-2	1:200	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
124	mouse IgG ₁	Bcl-2	1:80	DAKO A/S, Glostrup, Denmark
PC66	rabbit IgG	Bax	1:750	Oncogene Research Products, Cambridge, MA, USA

Immunocytochemistry and antibodies

The sections and cytopins were dried at room temperature for at least 1 h, fixed in acetone (at -20°C) and processed for immunohistochemical staining.

For the single immunostaining procedure, the slides were incubated overnight at 4°C with the primary antibodies. All incubations were carried out in phosphate-buffered saline (PBS) pH 7.4 at the appropriate dilution (table 1). The following day the slides were washed with PBS and incubated at room temperature for 30 min with the appropriate immunofluorescent-labeled secondary antibody (table 1). After extensive washing, the slides were mounted with a 4',6-diamidino-2-phenylindole (DAPI)-containing mounting agent (Vector Laboratories Inc., Burlingame, CA, USA) and stored at -20°C .

For double-immunostaining, the primary antibodies were selected on the basis of their isotypes, or a combination of monoclonal (mouse source) and polyclonal (rabbit source) antibody was applied as described in table 1. The first primary antibody was incubated overnight at 4°C and the next day, after washing, the second primary antibody was incubated at room temperature for 2 h. After extensive washing in PBS, a mix of the appropriate secondary antibodies was applied and incubated for 30 min at room temperature. Subsequently the slides were washed in PBS, mounted as described above, and stored at -20°C .

Amplification of signal was achieved by incubation with biotinylated goat-anti-rabbit (BIO-GAR) or biotinylated goat-anti-mouse (BIO-GAM) and Avidin-Biotin complex (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) after incubation with the primary antibody. Detection of peroxidase activity and

simultaneous signal amplification was achieved by incubation with tetramethylrhodamine isothiocyanate (TRITC)-labeled tyramide or fluorescein isothiocyanate (FITC)-labeled tyramide¹⁸.

Results

HE staining of skin biopsies and plucked hair

Comparison of the hair follicles derived from skin biopsies and from plucked hairs using light microscopy revealed that most of the epithelial structures from the hair follicle remain attached to the plucked hair (Fig.1).

Figure 1: H&E staining of plucked hair. Most epithelial structures from the follicle remain attached to the plucked hair. Adjacent to the longitudinal section of the plucked hair (x10) the respective areas are shown at a higher magnification (x40).

Basal cell makers: CK14 and CK19

Cells positive for the basal cell markers CK14 (RCK107, LL002) were found in the basal cells throughout the whole length of the ORS of the hair follicle and in the basal cell layer of stratified epithelium of the epidermis, both in the skin biopsy samples and in the plucked hairs.

Figure 2: A) Details (x40) from a longitudinally sectioned hair follicle from skin biopsies double stained for CK19 in red and Bcl-2 in green. Two areas were found to be positive for both markers; i.e. the upper third (A) and lower third (B) of the hair follicle.

Figure 3: A) Bcl-2 and B) CK19 staining of cells, derived from plucked hair and cultured for 14 days. Next to cells that are positive for both markers (arrows), some cells are only Bcl-2 or CK19 positive.

Figure 4: Details (x40) from a longitudinally sectioned dermal papilla (A) and hair follicle (B) from skin biopsies double stained for Bax in red and Bcl-2 in green.

Figure 5: Longitudinally sectioned plucked hair (x10) double stained for Bcl-2 in green and Ki-67 in red. Higher magnifications (x40) of areas found to be positive for both markers are shown next to the respective areas of the hair follicle.

In the hair follicles from the skin biopsy, the basal keratinocyte cell marker CK19 (RCK108, LP2K) was found in the most peripheral cell layer in two distinct areas in the upper (Fig 2A) and lower third (Fig 2B) of the ORS, but not in the epidermis. In the plucked hair, the same regions were found to be positive for CK19 (Results not shown). In follicular cell cultures approximately one fifth of the cells were CK19 positive. These CK19-positive cells were mostly found as cell clusters, as shown in Fig. 3B.

A complete overlap of CK19 (RCK108) and CK14 (LL002) was found in the biopsy samples and in plucked hair, and in approximately 40% of the cultured cells (not shown).

(Anti-) Apoptosis markers: Bcl-2 and Bax

Bcl-2 (N-19) positive cells were found in the outermost cell layer throughout the whole length of the ORS of the hair follicle from skin biopsies, as well as in the basal layer of the epidermis. We also found Bcl-2 positive cells in the most peripheral cell layer of the whole length of the ORS of the plucked hairs. Bcl-2 positive cells were not found in the dermal papilla. Double staining for CK19 (LP2K) and Bcl-2 (N-19) revealed that CK19-positive cells were also positive for Bcl-2. However, not all Bcl-2-positive cells were positive for CK19 (Fig. 2A, B). In the follicular cell cultures subjected to double-staining techniques, some cells stained positive for both Bcl-2 and CK19, while others were positive for either Bcl-2 or CK19 (Fig. 3A,B). Figure 3B shows a typical cluster of CK19 positive cells, found in the cytopins of the cell cultures.

Bax (PC66)-positive cells were present in the internal part of the ORS both in the hair follicles from skin biopsies and in the plucked hair. Bax-positive cells were found in the dermal papilla in the hair follicles from skin biopsies (Fig. 4A) and in the most proximal part of the plucked hair (Fig. 4B). Almost no overlap (<5%) between Bcl-2 (124) and Bax (PC66)-positive cells was found in either sample. However, in follicular cell culture there was more overlap (30–40%) between Bcl-2- and Bax-positive cells (results not shown).

(Hyper-) Proliferation markers CK16 and Ki-67

CK16 (LL025)-positive cells were found in the distal internal part of the ORS of the hair follicles, both from skin biopsies and plucked hairs. No CK16 staining was observed in the most proximal part of the hair follicles, either from skin biopsies or plucked hairs. CK16-positive cells were found in the internal part of the ORS, in contrast to CK19-positive cells whose position was more peripheral. There was no overlap between CK16 and CK19 staining in the hair follicles from skin biopsies, plucked hairs or follicular cell culture. There was an overlap in the immunostaining patterns of CK14 (LL002) and CK16 (LL025) in the ORS of the hair follicles from skin biopsies and in the plucked hair, where CK16-positive cells were located. In follicular cell culture there was virtually a total overlap between CK14- and CK16-positive cells (not shown).

Ki-67-positive cells were sporadically found (Fig 5). Their location tends to be more toward the internal part of the ORS, although some were found near the external part of the ORS near the CK19-positive cells. Ki-67-positive cells were also found in the dermal papilla. Less than 1% of Ki-67-positive cells were found in follicular cell culture.

Discussion

Although plucked hair seems to contain all of the epithelial structures that are present in the hair follicle derived from skin biopsies, the transplantation of plucked hair does not result in normal hair growth, in contrast to hair-containing punch grafts¹. The fact that the structures of the hair follicle remaining in the skin can produce a normal hair, suggests that follicular stem cells are retained in the skin. However, when hair follicle preparations derived from skin biopsies are compared with preparations from plucked hair under a light microscope, it can be seen that the majority of the epithelial structures from the hair follicle remains attached to the plucked hair. The question thus arises whether or not follicular stem cells are extracted with the plucked hair, and whether the stromal tissue surrounding these epithelial structures is necessary for induction of hair growth. This follicular connective tissue has specific biochemical characteristics, such as the presence of PDGF-alpha and PDGF-beta receptors and versican^{19,20}, that seem to play a role in (embryonic) hair follicle development and cycling.

We based our search for the follicular stem cell population in plucked hair and cultures thereof on stem cell characteristics proposed in the recent literature⁹, i.e. the expression of CK19 and Bcl-2, in combination with the absence (or low levels) of Bax expression²¹, and an extremely low proliferation frequency²². In 1996, Michel et al.²³ suggested that CK19-positive cells in hair follicles represent stem cells. Furthermore, stem cells in general express Bcl-2^{24,25}. In normal skin, Bcl-2 is only expressed by a limited population of cells in the basal compartment, which can be regarded as the stem cell compartment²⁶.

An important finding in this study is that phenotypical characteristics of the hair follicle from skin biopsies are preserved in plucked hair, including the expression of CK19¹⁰ and Bcl-2¹⁶ in cells from two areas of the upper and lower third of the follicle. Positivity for CK19 and Bcl-2 corresponds to infrequent cell division in these areas, as concluded from the absence of Ki-67 staining. The fact that these cells are positive for Bcl-2 and CK 19, but Ki-67 and Bax negative, is a strong indication that they represent stem cells in the hair follicle. Our results also indicate that transient amplifying cells in the epidermis, which are expected to be Ki-67 positive, are largely Bcl-2 negative. All the markers found in the hair follicle from skin biopsies were also preserved in the follicular cell culture derived from plucked hair, including CK19 and Bcl-2, and the basal cell marker CK14. We therefore conclude that viable follicular stem cells can be obtained from plucked hairs. The low frequency of Ki-67 positive cells indicates that differentiation may have occurred in these cell cultures, which can also be concluded from the CK10 staining in a proportion of the cells (unpublished observation).

The observation that two distinct areas in the hair follicle from skin biopsies are positive for CK19 was also found by Commo et al.¹³. The fact that these areas are also Bcl-2 positive, and Bax negative, is a strong indication for two stem-cell sites. The question therefore arises whether or not both areas are necessary for hair growth induction. In humans, Kim et al.²⁷ found that the proximal part of the hair follicle cannot regenerate into a differentiated hair follicle, but the distal part of the follicle can, eventually resulting in a fully developed hair follicle²⁸.

Reynolds et al.²⁹ found that, although the dermal papillae of humans cannot induce new hair growth, the sheath of the lower part of the hair follicle can. These apparently contradictory results indicate that both proximal and distal areas of the hair follicle can induce hair growth, which agrees with our finding of two stem cell locations.

In addition to these two areas, the dermal papilla has also been ascribed a key role in hair growth. In rats, cultured dermal papilla cells from whisker hairs can generate a fully differentiated hair follicle³⁰, suggesting that follicular stem cells are located in or near the dermal papilla. The cells in this region that are held responsible for hair growth are the germ cells. Recently a hypothesis of hair cycling was proposed that involves participation of these germ cells next to the bulge region stem cells³¹. In our study, the dermal papilla in the hair follicle of the skin biopsy did not contain Bcl-2. In contrast other studies have found Bcl-2 positivity in the dermal papilla^{16,32}, but the tissues used in these studies were either embryonic or derived from non-melanoma skin cancers. The apparent discrepancy in findings could be due to the fact that, although Bcl-2 is normally found in the basal compartment of normal skin, there is an expansion of this Bcl-2 positive cell population under pathological circumstances such as basal cell carcinoma (BCC)^{26,33}.

In our study we found that the dermal papilla is positive for Ki-67 and Bax. This immunophenotype, in combination with the Bcl-2 negativity, is not in agreement with the characteristic definition of a follicular stem cell population, but rather supports the findings of others that follicular stem cells are not present in the dermal papilla^{15,34}. However, taken all these findings together suggests that hair growth involves the co-operation of various stem cell regions.

The inability to successfully transplant plucked hairs containing the two stem cell regions may be due to the lack of appropriate conditions in the stromal tissue of the recipient areas. A similar lack of growth has been observed in case of transplanted epithelial structures derived from BCC which could not be induced to proliferate without the transplantation of additional stromal cells³⁵. This comparison is justifiable since BCC are most likely derived from follicular germinative cells^{36,37}. Another indication of the importance of stromal cells for the growth of keratinocytes is the observation that keratinocyte cultures may require a fibroblast feeder layer³.

Unsuccessful regeneration after implantation of plucked hairs may also be caused by the inflammatory response of the receptor area to the plucked hairs, which may result in the destruction of the follicular stem cells. The connective tissue surrounding these epithelial structures in micro-punch grafts may be crucial for protection against inflammation, and thus a successful transplantation. Therefore, the importance of the connective tissue of the hair follicle cannot be ignored^{1,38}. Follow-up studies will focus on the role of components of the connective tissue and the extracellular matrix surrounding the epithelial portion of the hair follicle.

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Chapter 3: Bcl-2 Family

3.1: Bax expression and growth behavior of basal cell carcinomas

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Abstract

Background

To understand the typical growth behavior of basal cell carcinoma (BCC) we searched for the correlation between proliferation and apoptosis and progression of BCC.

Methods

Expression of Bcl-2, Bax, and Ki-67 was immunohistochemically investigated in both normal skin and BCC cells, as well as in the epidermis overlying BCC.

Results

The results showed that in normal epidermis, Bcl-2 was homogeneously expressed in the basal cell compartment, whereas Ki-67 expression was largely restricted to the parabasal layer, the layer just above the basal cell layer, and exhibited a more scattered staining pattern. Bax was occasionally expressed in the basal layer and widely in the suprabasal compartment. Strikingly, the apparently normal epidermis overlying BCC showed an increased Bcl-2 staining. In BCC, cells stained homogeneously for Bcl-2, whereas Bax and Ki-67 showed scattered staining patterns. Simultaneous expression was seen for Bcl-2 and Bax in $80\pm7\%$ of the tumor cells, and co-expression of Bcl-2 and Ki-67 in $20\pm7\%$ of the tumor cells. The cells expressing Bcl-2 and Ki-67, but lacking expression of Bax, the progressive fraction, comprised on average $7\pm3\%$ of the tumor cell population.

Conclusion

These results suggest that this small progressive fraction of tumor cells, in combination with the relatively high percentage of cells still prone to apoptosis, can explain the indolent growth behavior of BCC.

Keywords: basal cell carcinoma, proliferation, apoptosis, Bax, Bcl-2, Ki-67

Introduction

Basal cell carcinoma (BCC), the most common malignancy in Caucasians, is a non-melanoma type of skin cancer arising predominantly in the face and neck. It is a typically slowly growing tumor with a great diversity in histopathological appearance¹. Although generally of indolent nature, occasionally the tumor can become very aggressive and destructive, a clinical behavior that is mainly related to the morpheiform, metatypical or micronodular phenotype¹.

In general, growth of a tumor is based on a derailment of cell proliferation². However, it has been shown in previous studies that the duration of a cell cycle in BCC resembles that of keratinocytes of the generative compartment of normal epidermis, approximately 217 hours³. This is in contrast with the clinical observation that BCC is an extremely slowly growing tumor, which may take months or even years to double in size⁴. It has therefore been suggested that only a small percentage of all tumor cells is actively proliferating and it was found that these cells are mainly located at the periphery of the tumor nests^{5,6}. In addition, an increase of the apoptotic potential of BCC cells may be another explanation for this phenomenon.

Apoptosis is partly regulated by the balance between the expression of anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax (Bcl-2 associated x-protein). The Bcl-2 gene was first discovered in B-cell-lymphomas showing a t(14:18) translocation⁷. The resulting fusion gene yielded an over-expression of the Bcl-2 protein⁸. This translocation is frequently found in various cancers and it was shown by Reed *et al*⁹ that the Bcl-2 protein could suppress apoptosis induced by various stimuli. Bax was discovered in an immunoprecipitation assay when it co-precipitated with Bcl-2. Although it exhibits a high degree of homology with Bcl-2, this protein showed pro-apoptotic activity¹⁰. Over-expression of Bax results in homodimerization and suppression of the functionality of Bcl-2, followed by the induction of apoptosis. Over-expression of Bcl-2, however, results in cell survival. It can thus be concluded that the relative expression levels of these two proteins in part determine the fate of a cell¹⁰. Since most tumor cells in BCC show expression of Bcl-2¹, the expression level of the Bax protein may determine whether a cell lives or dies.

The slow expansive growth of BCC must therefore be explained in the light of the delicate balance between cell generation (proliferation) and cell loss (apoptosis), which is responsible for the tissue homeostasis in multi-cellular organisms, and becomes disturbed in tumor development.

The aim of this study was to determine and quantify the cell population in BCC that is responsible for its expansion i.e. the tumor cells that are protected from undergoing apoptosis (Bcl-2 positive, Bax negative) and are actively proliferating (Ki-67 positive). It was found that this cell population comprises a minor fraction of the tumor cells and is smaller than the cell fraction that can potentially undergo apoptosis.

Figure 1: Specificity testing of the Bax and Bcl-2 antibodies

A. Polyclonal Bax antibody (Ab-1) in normal skin. Note sporadic expression in basal layer of the epidermis and more extensive expression in the suprabasal layers.

B. Polyclonal Bax antibody (Ab-1) pre-incubated with blocking peptide: no staining present.

C. Polyclonal Bcl-2 antibody (N-19) in BCC: epidermis and tumor cells show expression of the Bcl-2 protein.

D. Polyclonal Bcl-2 antibody (N-19) pre-incubated with blocking peptide: no staining present.

E. Monoclonal Bcl-2 antibody (Clone 100) in normal skin: basal layer positive, suprabasal layers negative.

F. Polyclonal Bcl-2 antibody (N-19) in normal skin: comparable result as observed for the monoclonal antibody.

G. Immunoblotting results with the monoclonal (Clone 124, lane a) and polyclonal antibody (N-19, lanes b and c) against Bcl-2: a single band is detected with both antibodies at the expected molecular weight of 29 kDa in the MCF-7 and Jurkat cell lines.

H. Immunoblot with the polyclonal Bax antibody (Ab-1): one single band was detected at the expected molecular weight of 21 kDa in 3T3, HT29 and HeLa cell lines.

The 40x objective was used for photography of images 1 A-F.

Figure 2: Proliferation- and apoptosis markers in the epidermis

A. Bcl-2 (green) and Ki-67 (red): Bcl-2 was found to be present in the basal cell layer of the epidermis of normal skin, whereas Ki-67 is mainly restricted to the parabasal layer.

B. Bcl-2 (green) and Ki-67 (red): There are some clear differences in expression of Bcl-2 and Ki-67 in the epidermis overlying BCC as compared to the expression patterns in normal epidermis. The expression is enhanced and simultaneous expression of the two markers occurs.

C. Bcl-2 (green) and Bax (red): Bax shows a low level of expression in the basal cell layer but is upregulated in the suprabasal layers of the epidermis. The expression patterns of Bcl-2 and Bax are mutually exclusive.

D. Bcl-2 (green) and Bax (red): A clear difference between the expression patterns of Bcl-2 and Bax in epidermis overlying BCC and normal epidermis is that simultaneous expression of Bcl-2 and Bax can occur in some parabasal cells (see arrows).

E. Bax (red) and Ki-67 (green): The expression patterns of Bax and Ki-67 can show overlap in some

parabasal cells (see arrows).

F. Bax (red) and Ki-67 (green): Increased number of cells showing Ki-67 expression in the epidermis overlying a tumor as compared to normal epidermis.

The 40x objective was used for photography of images 2 A-F.

Figure 3: Proliferation- and apoptosis markers in the BCC:

A. Bcl-2 (red) and Ki-67 (green): BCC stains homogeneously positive for Bcl-2, while Ki-67 yields

positive staining at the periphery of the nodules. Morpheic strands show a more scattered pattern of Ki-67 expression.

B. Bcl-2 (green) and Bax (red): BCC express Bcl-2 homogeneously, while Bax expression is found in 60-90% of the tumor cells.

C. Bax (red) and Ki-67 (green): Simultaneous expression occurs in tumor cells (white arrows), while also tumor cells are positive for Ki-67 but negative for Bax (yellow arrows).

The 40x objective was used for photography of images 3 A-C.

Materials and methods

Tissue material

Normal skin was obtained from breast reduction surgery at the Department of Plastic Surgery of our hospital and was snap frozen in liquid nitrogen. Frozen sections (5µm) were cut and placed on slides coated with 3-aminopropyltriethoxysilan (APTS) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

Fresh frozen tumor material was obtained from 30 BCC patients treated by Mohs' micrographic surgery. The histological classification of the BCCs showed that twelve were nodular type, three were micronodular type, one was morpheaform type, two were nodular/morpheaform type, six were micronodular/morpheaform type, two were nodular/adenoid type and four were mixed type (Table 3). In all these cases the apparently normal overlying epidermis was also examined. Frozen sections (5µm) were cut and placed on APTS-coated slides.

Methods

- Immunohistochemistry

Specificity of the polyclonal antibodies was warranted by the fact that they are both affinity purified using the synthetic peptide used to immunize the rabbits. Furthermore, immunoblotting was performed for the monoclonal- and polyclonal antibody against Bcl-2 and the polyclonal antibody against Bax as was described previously¹¹, using the following cell lines: HeLa (human cervix adenocarcinoma), HT29 (human colorectal adenocarcinoma), 3T3 (mouse fibroblast), MCF-7 (human adenocarcinoma of the mammary gland), and Jurkat (human T cell leukemia) (ATCC in Manassas, VA, USA). In addition, the polyclonal antibodies against Bax and Bcl-2 were pre-absorbed with the peptides used for immunization.

- Immunoperoxidase staining

Single antigen detection was performed using the immunoperoxidase staining method¹². Serial frozen sections were fixed in acetone and endogenous peroxidase activity was quenched by incubating the slides with 0.3% hydrogen peroxide in phosphate buffered saline (PBS). Nonspecific binding was blocked by incubation with normal goat serum. The slides were then incubated overnight at 4°C with the primary antibodies in optimal dilutions (Table 1).

The bound primary antibodies were linked with biotinylated secondary antibodies. The sections were then incubated with Avidin-Biotin complex horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA, USA). Peroxidase activity was detected with the chromogen 3,3'-diaminobenzidine (DAB) and the sections were counterstained with haematoxylin (Fluka A.G., Buchs, Germany).

- Double-immunofluorescence staining

Serial frozen sections were fixed in acetone and nonspecific binding was blocked by incubation with normal goat serum. The slides were then incubated

with the primary antibodies, either subsequently or simultaneously, overnight at 4°C. Table 2 shows the combinations of antibodies that were used.

For combinations A and B (Table 2) the overnight incubation was followed by incubation with peroxidase-conjugated goat-anti-mouse IgG (DAKO A/S, Glostrup, Denmark) and subsequently incubation with a mixture of Texas Red-labeled goat-anti-rabbit Ig (ITK Diagnostics, Uithoorn, The Netherlands) and fluorescein isothiocyanate (FITC)-labeled tyramide¹³ to detect peroxidase activity and simultaneous signal amplification.

For combinations C, D and E (Table 2), the slides were incubated with a mixture of FITC-labeled goat-anti-mouse Ig and Texas Red-labeled goat-anti-rabbit Ig.

For combination F, overnight incubation with the polyclonal Bax antibody was followed by incubation with biotinylated goat-anti-rabbit and Avidin-Biotin complex (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA). Detection of peroxidase activity and simultaneous signal amplification was achieved by incubation with tetramethylrhodamine isothiocyanate (TRITC)-labeled tyramide¹³. The slides were then incubated with the polyclonal Bcl-2 antibody and subsequently with FITC-labeled goat-anti-rabbit Ig (ITK Diagnostics, Uithoorn, The Netherlands).

Determination of percentages of positive cells

The percentages of positive cells were estimated by counting 500 cells per tumor by two independent observers.

Table 1: Antibody characteristics and dilutions used in the single label immunoperoxidase staining procedure

Antibody	Epitope	Manufacturer
Clone 100 (monoclonal)	Bcl-2 aa 44-52	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Clone 124 (monoclonal)	Bcl-2 aa 41-54	DAKO A/S, Glostrup, Denmark
N-19 (polyclonal)	Bcl-2 N-terminal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
P-19 (polyclonal)	Bax N-terminal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Ab-1 (polyclonal)	Bax aa 150-165	Oncogene Research Products, Cambridge, MA, USA
MIB-1 (monoclonal)	Ki-67	Immunotech, Marseille, France
A0047 (polyclonal)	Ki-67	DAKO A/S, Glostrup, Denmark

Abbreviation: aa = amino acids

Table 2: Combinations of primary antibodies applied in the double-immunofluorescence assay

Combination	Antibodies and dilutions
A	Clone 100 (1:10), N-19 (1:200), simultaneous incubation
B	Clone 124 (1:50), A0047 (1:200), simultaneous incubation
C	N-19 (1:200), MIB-1 (1:25), simultaneous incubation
D	MIB-1 (1:25), P-19 (1:200), simultaneous incubation
E	Clone 124 (1:50), P-19 (1:200), simultaneous incubation
F	P-19 (1:1000), N-19 (1:200), subsequent incubation

Results

Specificity testing of the immunoreagents

The specificity of the polyclonal- and monoclonal antibodies was warranted by several tests. Firstly, the polyclonal antibodies against Bax and Bcl-2 were pre-absorbed with the peptides used for immunization, resulting in absence of staining when the corresponding peptide was used (Fig. 1A-D). Secondly, in normal epidermis, the monoclonal antibody against Bcl-2 showed the same expression pattern of the protein as seen with the polyclonal antibody (Fig. 1E and F, respectively). In addition, the results of the immunoblotting showed the expected 29 kDa band for both the monoclonal- and the polyclonal antibody against Bcl-2 (Fig. 1G). Furthermore, the expected 21kDa band for the polyclonal antibody against Bax was seen (Fig. 1H).

Proliferation- and apoptosis markers in the epidermis

In normal skin, the expression of the Bcl-2 protein was found to be restricted to the basal cell layer of the epidermis (Fig. 2A). This distribution pattern was seen with the monoclonal as well as the polyclonal antibodies. Ki-67 expression was found to be predominantly restricted to cells present in the parabasal layer, the layer just above the basal cell layer (Fig. 2A). These cells were either scattered throughout this layer, or clustered in certain areas. Again the polyclonal and monoclonal antibodies gave rise to the same results. In general, the staining patterns of Ki-67 and Bcl-2 were mutually exclusive, but occasionally some basal cells showed simultaneous expression. Bax was found to be expressed in the suprabasal layers of the epidermis, and in an occasional basal cell. The double immunofluorescence studies revealed also mutually exclusive expression of Bcl-2 and Bax proteins in normal skin (Fig. 2C). Bax and Ki-67 occasionally showed co-expression in the normal epidermis (Fig. 2E).

We noticed some striking differences in the epidermis overlying BCC as compared to the epidermis of normal skin. Epidermis overlying BCC showed increased numbers of cells positive for Ki-67, with suprabasal layers of the epidermis also showing positive cells. With the monoclonal Bcl-2 antibody a staining pattern similar to that in normal skin was seen, but when using the polyclonal Bcl-2 antibody also para- and suprabasal cells showed positive staining. As a result, cells with overlapping expression of Bcl-2 and Ki-67 were shown (Fig. 2B). Also Bax and Bcl-2 could be detected simultaneously in the

suprabasal layers of the epidermis overlying BCC (Fig. 2D). As in normal epidermis, the Bax and Ki-67 staining patterns in the epidermis overlying BCC occasionally showed co-expression (Fig. 2F).

Proliferation- and apoptosis markers in BCC (Table 3)

A homogeneous staining pattern was found for Bcl-2 in BCC throughout the entire lesion, both for the polyclonal- and the monoclonal antibody. Expression of Ki-67 was shown in the tumor cells at the periphery of most of the nodules in the nodular type of BCC, and in a somewhat more variable pattern in the morpheaform type of BCC. The percentages of Ki-67 expressing cells varied greatly amongst the various histological types and within an individual tumor (6-32%) with an average of almost 20% (Table 3). Areas with a high number of cells expressing Ki-67 occurred however adjacent to areas with a lower number of Ki-67 positive cells (Fig. 3A)

The expression of the Bax protein showed a scattered staining pattern which varied among the different types of BCC and within the individual tumors (Fig. 3B), but on average it was found in approximately 80% of the tumor cells. The percentages of cells showing simultaneous expression of Bax and Ki-67 varied widely (4-25%) between tumors (Table 3 and Fig. 3C), and a correlation with the histopathological type could not be found. The proliferative (Ki-67 positive) fraction of the tumor consisted of cells either expressing or not expressing Bax. It is shown in Figure 4 that within the proliferative compartment of each individual tumor the percentage of Bax positive cells was higher than the percentage of Bax negative cells.

Figure 4: Bax status of the proliferative compartment

The graph represents the percentage of Bax positive and negative cells within the Ki-67 positive fraction. From this graph it can be seen that, in the proliferative compartment of each individual tumor the percentage of Bax positive cells is higher than the percentage of Bax negative cells.

Table 3: Frequency of expression of proliferation- and apoptosis markers in BCC

Subtype	Patient number	Ki-67 +	Bax +	Ki-67+ /Bax-	Ki-67+ /Bax+
Nodular	1	9%	82%	3%	4%
Nodular	2	19%	87%	7%	11%
Nodular	3	27%	85%	10%	19%
Nodular	4	26%	83%	8%	17%
Nodular	5	22%	74%	4%	16%
Nodular	6	6%	77%	2%	3%
Nodular	7	17%	82%	8%	10%
Nodular	8	24%	82%	8%	16%
Nodular	9	23%	87%	7%	16%
Nodular	10	25%	80%	9%	15%
Nodular	11	8%	70%	3%	5%
Nodular	12	27%	82%	4%	21%
Nod/morph	13	21%	87%	5%	15%
Nod/morph	14	14%	81%	4%	13%
Nod/aden	15	23%	67%	12%	12%
Nod/aden	16	23%	77%	8%	14%
Morphea	17	32%	83%	10%	20%
Mnod/morph	18	25%	60%	4%	25%
Mnod/morph	19	32%	72%	6%	16%
Mnod/morph	20	20%	78%	6%	16%
Mnod/morph	21	14%	79%	5%	9%
Mnod/morph	22	27%	80%	8%	19%
Mnod/morph	23	22%	79%	4%	19%
Micronodular	24	12%	88%	3%	10%
Micronodular	25	15%	79%	6%	8%
Micronodular	26	14%	87%	5%	8%
Mixed	27	23%	65%	11%	12%
Mixed	28	18%	76%	8%	12%
Mixed	29	12%	88%	4%	8%
Mixed	30	17%	77%	8%	16%
Mean %		19.7%	80.1%	6.4%	13.0%
SD		6.8%	7.0%	2.6%	5.2%

Abbreviation: Nod/morph = nodular/morpheaform, Nod/aden = nodular/adenoid
Mnod/morph = micronodular/morpheaform, SD = standard deviation

Based on the different percentages of cells staining positive for the three markers we can distinguish three different tumor cell fractions (Table 4). The progressive fraction, which stains positive for Bcl-2 and Ki-67, but is negative for Bax, accounts for $6.4 \pm 2.6\%$ of the tumor cells. This fraction of cells may be thought of as being responsible for the growth of the tumor since it is actively proliferating and protected from undergoing apoptosis. The fraction of cells that expresses all three markers may be regarded upon as proliferating but still prone to apoptosis because of the presence of Bax. This fraction comprises $13.0 \pm 5.2\%$ of the tumor cells. The third fraction ($47 \pm 6.2\%$), expressing Bax and Bcl-2, but lacking expression of Ki-67, is the non-proliferating fraction that is still prone to apoptosis.

Table 4: Frequency of different cell populations in BCC

Cell fraction	Marker pattern	Percentage
Progressive	Bcl-2+/Ki-67+/Bax-	$6,4 \pm 2,6$
Proliferative, prone to apoptosis	Bcl-2+/Ki-67+/Bax+	$13,0 \pm 5,2$
Non-proliferative, prone to apoptosis	Bcl-2+/Ki-67-/Bax+	$47,6 \pm 6,2$

Discussion

The growth rate in neoplastic lesions is often determined by both an increase in the fraction of proliferating cells, as well as a decrease in the rate of cell loss¹⁴. It has become apparent that in BCC an imbalance between apoptosis and proliferation may explain its progression. Candidates for regulating the rate of cell death are Bcl-2, which prevents apoptosis¹⁵⁻¹⁹, and Bax, which is known to induce apoptosis^{18,20}. These two proteins can form homo-dimers and hetero-dimers, and their balance eventually determines whether the process of apoptosis is induced or suppressed¹⁰. To detect cell proliferation, Ki-67 antibodies are most widely used. They bind to a nuclear antigen expressed by cycling cells^{21,22}.

Our results in normal skin are in concert with previously described studies, showing Bcl-2 expression in the basal layer, Ki-67 expression in the parabasal layer and Bax staining in a few basal cells, but predominantly in the suprabasal layers^{23,24}. Our double-label immuno-assays clearly demonstrated the almost exclusive staining patterns for Ki-67 and Bcl-2 on the one hand, and for Bcl-2 and Bax on the other. A few cells, which are most likely responsible for the regeneration of the epidermis, expressed both Ki-67 and Bcl-2. The expression of Bax in the suprabasal compartment may reflect the commitment of these cells to undergo terminal differentiation and eventually apoptosis, which also explains the downregulation of Bcl-2 in these cells.

The expression of these markers in the epidermis overlying BCC has not been studied extensively before. Our results showed Bax expression comparable with that in normal epidermis, but expanded staining with the polyclonal Bcl-2 antibody in the epidermis overlying BCC. The differences in staining patterns obtained by the monoclonal and polyclonal Bcl-2 antibodies may be explained

by the recognition of different epitopes. The epitope recognized by the monoclonal Bcl-2 antibody is apparently shielded in the suprabasal cell layers by heterodimerization of Bcl-2 with other proteins, for example Bax. Similar observations of epitope shielding have been described by Schandl *et al*²⁵.

The fraction of parabasal cells staining for Ki-67 was increased in the epidermis overlying BCC as compared with that in the epidermis of normal skin, while also more suprabasally located cells were Ki-67 positive. This suggests loss of cell cycle control and maintenance of the Bcl-2/Bax status in the process of migration of basal cells towards the suprabasal layer in the epidermis overlying BCC. A significantly increased Ki-67 expression was also detected in the hyperplastic epidermis overlying a keratotic melanocytic nevus, whereas Bcl-2 and Bax were expressed similarly in both normal and hyperplastic epidermis²⁶. This demonstrates increased proliferation in the context of adequately regulated apoptosis.

A similar mechanism may explain the phenotype of BCC, in which a cell fraction occurs which express both Bcl-2 and Ki-67, thus representing the actively proliferating fraction. The percentage of these cells, which are double positive for Ki-67 and Bcl-2, varied widely between tumors (6-32%) and also within the individual tumors. A correlation between the histological type of the tumor and the percentage of Ki-67 positive cells could not be found. Nevertheless, it may be obvious that these double-positive cells play a key role in the progression of the tumor, one reason being that they are prone to accumulation of genetic modifications.

A comparison between the growth behavior of trichoepithelioma (TE), a benign cutaneous tumor that originates from hair follicles²⁷, and BCC shows that BCC and TE exhibit both differences in Ki-67 and Bcl-2 expression^{28,29}. TE is a tumor characterized by proliferation of keratinocytes that rapidly differentiate into mature follicular cells, and which apparently cease to express Bcl-2. BCC is composed of a population of basaloid keratinocytes, which largely retain their ability to produce Bcl-2²⁹. Furthermore, the number of Ki-67 expressing cells is significantly higher in BCC²⁸, which may explain the differences in growth behavior between these tumors. The consistent lack of expression of Bcl-2 in squamous cell carcinoma (SCC), a common malignant neoplasm that arises in the skin, contrasts with the staining seen in BCCs and suggests that these malignancies do not share the same cells of origin. Furthermore, their difference in expression of Bcl-2 reflects their difference in regulation of cell turnover³⁰.

In BCC cells, a variable Bax expression pattern was found. The relatively large fraction of the cells expressing Bax in the tumor seems rather unexpected because this protein is known to act as a tumor suppressor by enabling cell loss through apoptosis¹⁰. Previous studies on the expression of Bax in BCC described conflicting results. Rossen *et al*³¹ showed that the protein is not expressed in BCC, whereas Delehedde *et al*²³ demonstrated that the Bax protein is in fact expressed in BCC. We found an expression pattern similar to that of Delehedde *et al*²³, using the same antibody as used by Rossen *et al*²⁵. However, we used frozen tissue sections, while these authors used paraffin embedded material, which may explain the different results.

It was shown in previous studies^{6,32,33} that, based on morphology, the frequency of apoptotic cells in different types of BCC ranged from 0.6% to 51%. This corroborates our findings that approximately 80% of the BCC cells express Bax

and is therefore prone to apoptosis. This relatively high percentage of Bax expressing cells, combined with the relatively low fraction of the cells expressing both Ki-67 and Bcl-2, may explain the slow progression rate of BCC. In line with our finding that a relatively large proportion of the BCC cells express the Bax protein, Mooney *et al*⁶ described that the *apoptosis: mitosis* ratio for BCC was found to be 15.1, as compared to 2.1 for melanoma. Although the finding by several authors that up to 50% of the BCC cells can be apoptotic^{6,26,27}, is now explained by the high percentages of cells expressing Bax. The question remains whether Bcl-2 is still functional. Previous studies already mentioned Bcl-2 mutations. Huang *et al*³⁴ showed that, when the serine residue at position 70 of the Bcl-2 protein is altered into an alanine residue, the anti-apoptosis function of the protein is diminished because the protein cannot be dephosphorylated. Other inactivating mutations were deletion of the conserved BH4 domain, as well as G145E and W188A mutations³⁴. Furthermore, Uhlmann *et al*³⁵ showed that the deletion of a non-conserved region between residues 51 and 85 of human Bcl-2 resulted in an enhanced interaction with the death-promoting protein Bax, but no correlation with the ability of Bcl-2 to interact with other proteins was observed.

In summary we can state that the results of our study show a relatively small progressive cell fraction in BCC that is protected from apoptosis and actively proliferating. The failure of these cancer cells to undergo programmed cell death may contribute substantially to tumor progression. It has however also become clear that a relatively high percentage of BCC cells express considerable levels of Bax, a pro-apoptotic protein. In particular the presence of this component in BCC may explain its indolent growth behavior.

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3.2: Expression of pro- and anti-apoptotic members of the Bcl-2 family and associated proteins in normal human skin and basal cell carcinoma

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Abstract

Background

Programmed cell death (apoptosis) is an important step in tissue homeostasis, with members of the Bcl-2 protein family regulating this process. The delicate balance between pro- and anti-apoptotic members of this family is disturbed in several cancers.

Methods

Expression of the pro-apoptotic proteins Bid, Bad and Bcl-xs, activated caspase-3, and the anti-apoptotic proteins Bag-1, Bcl-xl and 136SerP-Bad, and two 14-3-3 variants known to interact with 136SerP-Bad, were immunohistochemically studied in normal skin and basal cell carcinoma (BCC), as well as in the apparently normal epidermis overlying BCC.

Results

In normal skin the basal layer of the epidermis stained homogeneously positive for Bag-1, Bcl-xs, Bcl-xl, Bid and Bad, whereas the suprabasal cell layers remained negative for Bag-1, Bcl-xs and Bid, while Bcl-xl and Bad were present in the entire epidermis. 136SerP-Bad and cleaved caspase-3 showed a scattered suprabasal pattern of expression in normal epidermis. 14-3-3 ϵ showed a homogeneous expression pattern in the entire epidermis, while 14-3-3 σ was absent from the basal cell layer. The majority of tumors showed homogeneous cytoplasmic staining for Bag-1, Bcl-xs, Bcl-xl, Bid and Bad, whereas 136SerP-Bad was found in less than 10% of the tumor cells in all cases and cleaved caspase-3 in less than 5%. 14-3-3 ϵ was homogeneously expressed in the tumors, while 14-3-3 σ was predominantly expressed in the central part of the tumors. The apparently normal epidermis overlying BCC showed enhanced expression patterns for Bcl-xs, Bid, Bag-1 and 14-3-3 σ . The low frequency of tumor cells showing activated caspase-3 was often found to be localized in the central part of BCC tumor nests, which was mutually exclusive with 136SerP-Bad which was found to be expressed in the periphery of these tumor areas.

Conclusion

The pro- and anti-apoptotic markers studied show comparable patterns of expression in the basal cell layer of normal epidermis and in BCC. In the apparently normal epidermis overlying BCC, differences were seen in expression patterns as compared to normal epidermis. This altered expression may reflect a role of these constituents in the pathogenesis of BCC.

The presence of anti-apoptotic 136SerP-Bad positive cells in the peripheral cell layers of tumor areas may be the result of growth stimulating effect on these cells by surrounding survival factors. The central cells of these tumor nests are largely devoid of these factors and are therefore subject to apoptosis as indicated by caspase-3 activation.

Keywords: apoptosis, basal cell carcinoma, Bcl-2 family

Introduction

The disturbance of the delicate balance between cell proliferation and cell death, necessary for normal development and tissue homeostasis, may result in development and progression of cancer^{1,2}. We have recently reported a imbalance between cell death and cell proliferation in basal cell carcinoma (BCC) based on the immunohistochemical staining results for the anti-apoptotic Bcl-2 protein, the pro-apoptotic Bax protein and the proliferation marker Ki-67³. The study furthermore identified a small progressive cell population in BCC, which could explain its indolent growth behavior. However, in recent years, a large number of Bcl-2 related proteins have been isolated that serve as critical regulators of apoptosis, acting either to inhibit or to promote cell death^{4,5}. This family of proteins can be subdivided into three categories.

The first category consists of the anti-apoptotic members such as Bcl-2⁶ and Bcl-xl⁷ that share homology in four Bcl-2 sequence homology (BH) regions^{8,9}, i.e. BH1 to BH4⁸. The second category consists of pro-apoptotic members, such as Bax¹⁰ and Bak¹¹ that share sequence homology in BH1 to BH3, but not in BH4. The third category comprises the 'BH3-only proteins', such as Bim¹², Bid¹³ and Bad¹⁴, all with a pro-apoptotic activity¹⁵. Heterodimerization between these anti- and pro-apoptotic family members can occur¹⁶⁻¹⁸. The balance in the expression of Bcl-2 family members is an important determinant of the cells' fate. Furthermore, modification of the Bcl-2 family members, e.g. by phosphorylation, dephosphorylation or cleavage by activated caspases will determine their functional properties. Bad can become phosphorylated under the influence of several growth factors, upon which the protein interacts with 14-3-3 proteins and remains inactive in the cytosol¹⁹. Bid becomes cleaved by activated caspase-8²⁰ upon which the C-terminal fragment can translocate to the mitochondria to induce cytochrome C release²¹. Another protein associated with prolonged cell survival is Bag-1, which can bind to Bcl-2, enhancing its anti-apoptotic effect²². The potential contribution of these Bcl-2 family members to the pathogenesis of BCC is still largely unknown, although a possible role in other types of malignancies has been described²³⁻²⁷. To examine the potential contribution of these Bcl-2 family members and associated proteins to the pathogenesis of BCC, we immunohistochemically investigated their expression patterns in frozen sections of normal skin, apparently normal epidermis overlying BCC, and 24 cases of this malignancy. An imbalance between these pro- and anti-apoptotic constituents should result in apoptosis in a certain percentage of (tumor) cells. This fraction of cells is estimated using an antibody to activated caspase-3, an executioner caspase stimulated via different routes of apoptosis induction²⁸.

Materials and Methods

Tumor samples

Normal skin biopsies (n = 5) were obtained by breast reduction surgery at the Department of Plastic Surgery, and the BCC (n = 24) samples were obtained by Mohs' micrographic surgery at the Department of Dermatology of our hospital.

These specimens comprised 12 cases of the nodular type, 2 cases of the micronodular type, 5 cases of the morphea type, 3 cases of the nodular-morphea type, 1 case of the micronodular-morphea type, and 1 case of the mixed type. The samples were snap frozen in liquid nitrogen and stored at -80°C until used. Serial sections ($5\mu\text{m}$) were cut and placed on 3-aminopropyltriethoxysilan (APTS; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands)-coated slides. The histopathologic diagnosis was established using haematoxylin (Fluka AG, Buchs, Germany) and eosin (BDH Laboratory Supplies, Poole UK) staining.

Immunohistochemistry

After fixation in acetone (-20°C), the slides were incubated with the primary antibodies, overnight at 4°C . Table 1 shows the antibodies used in this study and the dilutions applied. The antibodies were diluted in 1% bovine serum albumin (BSA; Roche Diagnostics GmbH, Mannheim, Germany) in PBS to block non-specific binding.

The primary antibodies against Bag-1, Bcl-xl, Bcl-xs, 136SerP-Bad, 14-3-3 ϵ and 14-3-3 σ were detected with peroxidase-conjugated secondary antibodies depending on the isotype of the primary antibodies. Peroxidase activity was detected using the chromogen 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), and the sections were counterstained with haematoxylin. For the detection of Bid further enhancement of the signal was necessary. After incubation with the peroxidase-conjugated secondary antibody, the slides were incubated with biotin-conjugated tyramide (Dupont NEN, Boston, MA, USA), followed by incubation with peroxidase-conjugated avidin (DAKO A/S, Glostrup, Denmark). The peroxidase activity was then detected using DAB and the slides were counterstained using haematoxylin. Detection of Bad and activated caspase-3 required blocking of a-specific staining prior to incubation with the primary antibody. The slides were incubated using 5% normal goat serum (NGS)/PBS for 1 hour at room temperature. Following this incubation, the slides are incubated with the primary antibody overnight at 4°C . After incubation with the secondary antibody, the ABC-detection kit was used. The peroxidase activity was then detected using DAB and the slides were counterstained using haematoxylin. As a negative control for all antibodies, the primary antibodies were omitted.

Evaluation of the frequency of positive cells was as follows: - absence of staining; +/- staining in a small fraction of cells i.e. less than 10%; + staining in most tumor cells i.e. more than 75%; ++ homogeneous staining pattern.

Results

The staining results of the antibodies against Bcl-xs, Bcl-xl, Bid, Bad, 136SerP-Bad, 14-3-3- ϵ , 14-3-3- σ , Bag-1 and cleaved caspase-3 are summarized in Table 2 and depicted in Fig.1.

Table 1: Antibody characteristics and their dilutions used in the immunoperoxidase staining procedure

Antibody	Manufacturer	Dilution
Bad, polyclonal IgG	Cell Signaling Technology, Beverly, MA,USA	1:50
136SerP-Bad, polyclonal IgG	Santa Cruz Biotechnology Inc., Santa Cruz, CA,USA	1:50
Bag-1, polyclonal IgG	Santa Cruz Biotechnology Inc., Santa Cruz, CA,USA	1:200
Bcl-xl, monoclonal IgG2a	Zymed Laboratories Inc., San Francisco, CA,USA	1:50
Bcl-xs, rabbit polyclonal IgG	Oncogene Research Products, Cambridge, MA,USA	1:25
Bid, polyclonal IgG	Santa Cruz Biotechnology Inc., Santa Cruz, CA,USA	1:100
Activated caspase-3, polyclonal IgG	Cell Signaling Technology, Beverly, MA,USA	1:50
14-3-3 ϵ , monoclonal IgM	BD Transduction Laboratories, Heidelberg,Germany	1:250
14-3-3 σ monoclonal IgG1	Biocarta, San Diego, CA,USA	1:100

Figure 1: Expression patterns of pro- and anti-apoptotic members of the Bcl-2 family and associated proteins in skin and BCC. Original magnifications used for images A,B,D,E,G-K,M-O 20x, images C,F,L 40x.

Normal skin

In normal skin, the basal cell layer of the epidermis stained homogeneously positive for Bcl-xs (Fig.1A), Bcl-xl, Bid, Bad, 14-3-3- ϵ and Bag-1 (Fig.1J), whereas the suprabasal cell layers remained negative for Bcl-xs, Bid and Bag-1, while Bcl-xl, Bad and 14-3-3- ϵ were expressed homogeneously in the suprabasal cell layers. 136SerP-Bad (Fig.1D) was detected in a small fraction of cells in the epidermis, as was cleaved caspase-3 (Fig.1M). 14-3-3- σ (Fig.1G) was negative or present only in a small fraction of basal cells, but homogeneously expressed in the suprabasal cells.

Epidermis overlying BCC

Bcl-xs stained homogeneously positive in the basal layer of the epidermis overlying BCC in all of the 24 cases and was homogeneously positive in the suprabasal layers in 9 of the 24 cases (38%). Further 9 cases showed positive staining in the majority of suprabasal cells (Fig.1B), while 4 cases (17%) showed occasional immunoreactivity in the suprabasal cells. The remaining 2 cases (8%) remained negative in the suprabasal layers. Bcl-xl stained the entire epidermis homogeneously positive in all 24 cases. Bid staining was homogeneous in the basal cell layer of all 24 cases, 10 cases (42%) showed occasional immunoreactivity in the suprabasal cells, while the remaining 14 cases (58%) remained negative in the suprabasal cell layers. Bad was homogeneously expressed in all cell layers in all cases, whereas 136SerP-Bad (Fig.1E) was present in only a small fraction of epidermal cells in all cases. 14-3-3- ϵ was homogeneously expressed in the entire epidermis in all cases, whereas 14-3-3- σ (Fig.1H) is expressed in a small fraction of basal cells but homogeneously in the suprabasal cells in all cases. All 24 cases showed homogeneous cytoplasmic staining of the basal cell layer for Bag-1 and 10 cases (42%) showed negative staining in the suprabasal layers. However, 5 cases (21%) stained homogeneously positive in the suprabasal cell layers and 5 cases showed Bag-1 staining in the majority of suprabasal cells (Fig.1K). The remaining 4 cases (17%) showed occasional immunoreactivity for Bag-1 in the suprabasal cell layers. Cleaved caspase-3 is expressed in a small fraction of epidermal cells in all cases (Fig.1N).

Basal cell carcinoma

Bcl-xs staining resulted in a homogeneous cytoplasmic staining all BCCs studied (Fig.1C). Staining for Bcl-xl resulted in a homogeneous cytoplasmic staining in 19 of the 24 cases (79%), 2 cases (8%) showed partial positivity in some fields and homogenous staining in other fields, while one case showed only occasional positive staining in cells mainly located in the center of the tumor, and 2 cases remained negative. Bid staining resulted in a homogeneous cytoplasmic staining in 22 of the 24 cases (92%), while the remaining 2 cases showed either partial positivity, or a combination with a homogeneous staining in some fields. Bad was homogeneously detected in 18 cases (75%), while 6 cases showed immunoreactivity in the majority of cells. In contrast, 136SerP-Bad immunopositivity was detected in a small fraction (<10%) of the tumor cells (Fig.1F) in the majority of cases (71%), whereas the remaining tumors showed no expression of 136SerP-Bad. 14-3-3- ϵ was homogeneously expressed in all

cases, whereas 14-3-3 σ was present in the majority of tumor cells, predominantly located in the center of the tumor nests (Fig.11) in all cases. Bag-1 staining of the BCCs resulted in a homogeneous cytoplasmic staining (Fig.1L) in 23 of the 24 cases (96%), 1 case showing partial positivity in some fields and homogeneous staining in other fields. Cleaved caspase-3 was detected in a small fraction of tumor cells (approximately 5%) in all cases (Fig.1O) and was often predominantly located in the central part of the tumor nests.

Table 2: Expression patterns of pro- and anti-apoptotic members of the Bcl-2 family and associated proteins in skin and BCC

		Bcl-xs pro	Bcl-xl anti	Bid pro	Bad pro	P-Bad anti	14-3-3 ϵ	14-3-3 σ	Bag-1 anti	Capase-3
Normal Skin	basal cells	++ (5/5)	++ (5/5)	++ (5/5)	++ (5/5)	+/- (5/5)	++ (5/5)	-(3/5) +/- (2/5)	++ (5/5)	+/- (5/5)
	suprabasal cells	- (4/5) +/- (1/5)	++ (4/5) + (1/5)	- (5/5)	++ (3/5) + (2/5)	+/- (5/5)	++ (5/5)	++ (5/5)	- (5/5)	+/- (5/5)
Epidermis overlying BCC	basal cells	++ (24/24)	++ (24/24)	++ (24/24)	++ (24/24)	+/- (24/24)	++ (5/5)	+/- (24/24)	++ (24/24)	+/- (24/24)
	suprabasal cells	++ (9/24) + (9/24) +/- (4/24) - (2/24)	++ (24/24)	- (14/24) +/- (10/24)	++ (24/24)	+/- (24/24)	++ (5/5)	++ (24/24)	- (10/24) ++ (5/24) + (5/24) +/- (4/24)	+/- (24/24)
BCC	tumor cells	++ (24/24)	++ (19/24) + (2/24) +/- (1/24) - (2/24)	++ (22/24) + (1/24) +/- (1/24)	++ (18/24) + (6/24)	+/- (17/24) - (7/24)	++ (24/24)	+ (24/24)	++ (23/24) + (1/24)	+/- (24/24)

Abbreviations: pro = pro-apoptotic protein, anti = anti-apoptotic protein

-: negative

+/-: positivity in a small fraction of cells <10%

+: positivity in majority of cells >75%

++: homogeneously positive

(x/y): number of positive cases over total number of cases

Discussion

The development of cancer is accompanied by disturbances in cell cycle regulation as well as by the suppression of programmed cell death, i.e. apoptosis¹. In an earlier study³ we suggested that in BCC only a small population of tumor cells combines these features. We came to this conclusion on basis of the simultaneous expression of the proliferation marker Ki-67 and the anti-apoptotic Bcl-2 protein, in the absence of the pro-apoptotic Bax protein. The control of apoptosis is, however more complex, involving many more pro- and anti-apoptotic constituents and associated proteins^{4,9}. This study aims at establishing the role of the specific apoptosis-regulating members of the Bcl-2 family and associated proteins in skin carcinogenesis by studying their expression patterns in normal human skin and BCC through immunohistochemical assays.

As shown in this study a number of pro- and apoptotic members of the Bcl-2 family, i.e. Bag-1, Bcl-xs, Bid and as described before also Bcl-2³ are largely restricted to the basal cell compartment of the normal epidermis. In virtually all BCCs studied, these four constituents were found to be homogeneously expressed and to exhibit a cytoplasmic localization. Thus, the basal cell phenotype with respect to apoptosis regulating proteins is clearly reflected in BCC by these markers.

High expression of anti-apoptotic Bag-1 protein has in general been correlated with a predisposition for more aggressive behavior of the tumor and less favorable clinical outcome, probably by enhancing the anti-apoptotic activity of Bcl-2²². In oral squamous cell carcinomas, high Bag-1 expression was frequently correlated with nodal metastasis²⁹. Furthermore, over-expression of Bag-1 leads to progression of malignant melanoma and promotes metastasis to lung³⁰. In patients with invasive breast carcinomas, Bag-1 expression, mainly located at the nucleus, correlated with less favorable clinical outcome³¹. Laryngeal carcinoma patients furthermore showed that nuclear Bag-1 expression correlated with a significantly lower survival rate after radiotherapy³². Because of its apparent correlation with highly aggressive behavior, the relatively abundant expression of Bag-1 in BCC is quite surprising since these tumors in general exhibit an indolent clinical behavior. However, in our study Bag-1 showed a cytosolic localization in normal skin as well as in BCC, which may explain this apparent discrepancy.

Bcl-xl and Bcl-xs are both splice variants of Bcl-x⁷, Bcl-xs exerting pro-apoptotic and Bcl-xl anti-apoptotic effects. Whereas Bcl-xs is expressed only in the basal cells of normal skin, Bcl-xl is expressed homogeneously throughout the entire epidermis, and has been shown to be essential for protecting skin and other epithelial cells from apoptosis³³. Furthermore, it was shown that the Bcl-xl expression level increases from primary melanoma to melanoma-metastases, whereas Bcl-xs expression decreases during melanoma progression. This may reflect an increased malignant potential caused by an inhibition of apoptosis and growth advantage for metastatic melanoma cells³⁴. In our study, Bcl-xs expression was homogeneous in all BCCs, while 19 of the 24 cases showed also homogeneous expression of Bcl-xl in the tumors, indicating a delicate balance between these two Bcl-2 family members.

The pro-apoptotic protein Bid is normally activated by cleavage through caspase-8, after which the C-terminal fragment translocates to the mitochondria where it induces cytochrome C release²⁰. High levels of Bid expression were reported previously in locally advanced prostate cancer²³, where it was associated with longer recurrence-free survival. It was furthermore stated that the apoptosis-inducing Bid could possibly be of great importance in positive tumor responses to specific chemotherapeutic drugs. These studies and the underlying experiments can however not discriminate between intact Bid and its C-terminal fragment, because of the absence of antibodies specific for the latter.

Furthermore, the enhanced expression of Bag-1 in the suprabasal cell layers of the apparently normal epidermis overlying BCC, as compared to control epidermis, may indicate subtle pre-neoplastic changes that occur in this tissue. A similar result was obtained for Bcl-2 in our earlier study³ on BCC. The anti-

apoptotic effects of Bag-1 and Bcl-2 in this tumor overlying epidermis may however be (partially) compensated for by enhanced expression of pro-apoptotic Bcl-xs. These findings in skin overlying BCC, with apoptosis regulating proteins typically found in basal cells of normal skin, do however show that this apparently normal epidermis exhibits a phenotype in-between that of normal skin and BCC. A similar conclusion was drawn from studies on the expression of the proliferation marker Ki-67 and of the nuclear A-type lamins³⁵.

A remarkable result was found for the expression pattern of 14-3-3 σ in the epidermis of normal skin and BCC. In normal skin, the basal cell layer of the epidermis remains negative, a phenomenon also shown by the outer (basal) cell layers of the tumor nests. This supports the earlier hypothesis that the basal cell phenotype is clearly reflected in BCC.

Furthermore, the expression patterns of 136SerP-Bad and cleaved caspase-3 seem to be mutually exclusive in the BCCs we studied. Where cleaved caspase-3 positive cells are mainly located in the central part of the tumor nests, the 136SerP-Bad positivity is predominantly located at the periphery. This suggests, that the presence of surrounding survival factors results in survival and consequent progression of the peripherally located cells, whereas the central cells of these tumor nests are largely devoid from these factors and are therefore subject to apoptosis.

In conclusion we can state, that while BCC shows a remarkable basal cell like phenotype, the epidermis overlying BCC shows a phenotype in between that of normal skin and BCC. Whether this phenomenon is induced by factors generated by the BCC or can be regarded as a first step in skin carcinogenesis remains to be established. Furthermore, the 10% of tumor cells that are positive for 136SerP-Bad reflect the progressing fraction of the tumors and corroborate our earlier finding that approximately 6% of the tumor cells represent the progressive fraction³. We can thus conclude that several members of the Bcl-2 family indicated that the balance between anti- and pro-apoptotic proteins plays an important role in the progression of BCC.

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Chapter 4:

Nuclear lamins in the skin

Lamin Expression in Normal Human Skin, Actinic Keratosis, Squamous Cell Carcinoma and Basal Cell Carcinoma

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Abstract

Background

Aberrant expression patterns of nuclear lamins have been described in various types of cancer depending on the subtype of cancer, its aggressiveness, proliferative capacity and degree of differentiation. In general, the expression of A-type lamins (lamins A and C) has been correlated with a non-proliferating, differentiated state of cells and tissues.

Objectives

To establish and compare the expression patterns of lamins in normal human skin, actinic keratosis (AK), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC).

Methods

Expression patterns of the individual lamin subtypes were studied immunohistochemically. The proliferation capacity of the tumor cells was detected using a specific antibody to Ki-67, and was related to the A-type lamin expression patterns.

Results

In normal skin, lamin A was expressed in the suprabasal cell compartment of the epidermis, whereas the basal cells were mostly unstained. BCCs and SCCs stained positive in the most cells, while the epidermis overlying BCC and SCC and the epidermis in AK stained homogeneously and strongly in the basal cells in addition to the suprabasal cells. Lamin C was expressed in some basal cells of normal epidermis, while the suprabasal cells stained strongly positive. Both BCCs and SCCs stained strongly positive for lamin C, with the difference that in BCC the staining was predominantly present in nucleolar structures with occasional staining of the nuclear envelope. The epidermis overlying SCC showed strong positivity in the lamina of virtually all cells. The expression of lamin C in the basal cells of AK resembled the expression pattern seen in the epidermis overlying BCC, i.e. a nucleolar staining next to nuclear envelope staining. Lamin B1 and B2 were found in virtually all cells in normal epidermis, AK, BCCs, SCCs and the epidermis overlying cancer. The percentage of Ki-67 expressing cells was highest in BCC (45%), and gradually decreased via epidermis overlying BCC, AK, SCC, epidermis overlying SCC to normal skin (11%). Simultaneous expression of A-type lamins and Ki-67 occurred in approximately 50% of the proliferating (Ki-67 positive) cells in BCC and SCC.

Conclusion

Significant changes occur in the expression patterns of A-type lamins in both pre-malignant and malignant lesions of the skin. The profound overlap of lamin A and Ki-67 staining patterns indicates that the proliferating tumor cells may obtain a certain degree of differentiation. Finally, lamin A expression in the basal cell layer of the apparently normal epidermis overlying BCC may suggest its involvement in the primary process.

Keywords: actinic keratosis, basal cell carcinoma, epidermis, lamins, proliferation, squamous cell carcinoma

Introduction

Nuclear lamins are intermediate filament proteins that form a network at the nucleoplasmic site of the nuclear membrane. Two subtypes of nuclear lamins exist in humans, i.e. A-type lamins (also designated Statins), including lamin A, lamin A Δ 10 and lamin C, all three arising from the same gene by alternative splicing^{1,2}, and B-type lamins, i.e. lamin B1 and lamin B2, arising from two distinct genes^{3,4}. In general, the A-type lamins are expressed in differentiated cells and tissues, whereas B-type lamins are also expressed in undifferentiated cells. From previous studies it became clear that the expression of both A-type lamins and B-type lamins is differentially regulated in human tissues⁵ and during embryogenesis⁶.

Lamins share many characteristics with the other intermediate filament subclasses, but they are located exclusively in the nucleoplasm instead of the cytoplasm. A nuclear localization signal at the carboxy-terminus directs the transport into the nucleus⁷. Furthermore, the nuclear lamins form two-dimensional sheets underlying the nuclear membrane, while cytoplasmatic intermediate filaments form fibrous structures extending from nucleus to plasma membrane⁸.

Although many biochemical and molecular features have been studied, the function of the lamins remains largely undetermined. It can be assumed that nuclear lamins not only maintain the integrity of the nucleus and the position of nuclear pore complexes, but are also thought to be important in maintaining the interphase organization of chromatin⁹ and may be involved in the regulation of gene expression^{10,11}. Spann *et al*¹² showed that alteration of lamin organization inhibits transcription and thereby regulates gene activity.

Aberrant expression patterns of lamins have been described in cancer. The nuclear matrix may play a role in cancer development as a result of alterations in protein expression during transformation¹³. Earlier studies have shown that lamin expression in some tumors is dependent on the subtype of cancer, its aggressiveness, proliferative capacity and degree of differentiation¹⁴⁻¹⁶. In general, A-type lamin expression is correlated with a non-proliferating, differentiated state of cells and tissues⁶, and therefore one would expect an altered expression of A-type lamins in cancer. In basal cell carcinoma (BCC), Venables *et al*¹⁷ have recently demonstrated that the absence of lamin A correlated with rapid growth, while the absence of lamin C correlated with slow growth of this skin tumor.

Furthermore, proteolytic lamin degradation has been shown in apoptotic cells¹⁸ and this degradation seems to proceed DNA fragmentation¹⁹. The presence of artificially introduced degradation-resistant lamins delays the process of apoptosis²⁰, suggesting that the nuclear lamins play an important part in the process of apoptosis by facilitating nuclear events during apoptosis, such as chromatin condensation. Since differences exist in the fraction of apoptotic cells in squamous cell carcinoma (SCC) and BCC²¹⁻²⁴, a comparison was made of lamin staining patterns in these malignancies.

Thus, this study aims at establishing and comparing the expression patterns of lamins in normal human skin, actinic keratosis (AK), SCC and in BCC in order to investigate the role of lamins in the process of skin carcinogenesis.

Materials and methods

Tissues

Samples of normal human skin (n=5) were obtained from reduction surgery of human breast or abdomen. The BCC samples (n=15) were obtained from Mohs' micrographic surgery. The AK (n=5) and SCC (n=10) specimens were obtained from biopsies. All specimens were snap-frozen in isopentane and 5 µm sections were cut onto 3-aminopropyltriethoxysilan (APTS; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) -coated slides, air-dried and fixed in acetone (10 minutes, -20°C).

Table 1: Antibodies and their dilutions used in this study

Clone/Name	isotype	Antigen	Dilution	Reference/Source
41CC4	mouse IgM	Lamins A/C	ud	Burke <i>et al.</i> , 1983 ³⁶
R27	mouse IgM	Lamins A/C	ud	Zatloukal <i>et al.</i> , 1992 ³⁷
133A2	mouse IgG ₃	Lamin A	1/500	Abcam Ltd., Cambridge, UK ³⁰
RaLC	rabbit polyclonal	Lamin C	1/50	Abcam Ltd., Cambridge, UK ¹⁷
119D5F1	mouse IgG ₁	Lamin B1	1/250	Abcam Ltd., Cambridge, UK ²⁶
LN43	mouse IgG ₁	Lamin B2	ud	Abcam Ltd., Cambridge, UK ³⁸
X223	mouse IgG ₁	Lamin B2	ud	Research Diagnostics, NJ, USA ³
A0047	rabbit polyclonal	Ki-67	1/200	DAKO A/S, Glostrup, DK

Abbreviation: ud = undiluted

Immunohistochemistry

Table 1 shows the antibodies that were used in both immunoperoxidase and immunofluorescence staining procedures.

-Immunoperoxidase staining protocol:

All incubations were carried out at room temperature in a moist chamber. After incubation with the primary antibody for 1 hour, the slides were rinsed with phosphate buffered saline (PBS) (3x 5 minutes). Then, a secondary antibody (either goat-anti-mouse IgG (SBA, Birmingham, AL, USA) or swine-anti-rabbit IgG (DAKO A/S; Glostrup, DK), dependent on the primary antibody) conjugated with peroxidase, was used to incubate the slides for 1 hour (1:100 diluted in PBS). After rinsing with PBS the slides were then incubated with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Chemie BV), 1:10 diluted in PBS + 0,03% H₂O₂, for 15 minutes. The slides were then rinsed with water for 15 minutes. Counterstaining was carried out with haematoxylin (Sigma-Aldrich Chemie BV). After dehydration the slides were embedded with Entellan (MERCK, Darmstadt, Germany).

-Immunofluorescence staining protocol:

After overnight incubation at 4°C with the primary antibody, the slides were rinsed with PBS (3x 5 minutes). Then, a secondary antibody (dependent on the primary antibody either goat-anti-mouse IgG or goat-anti-rabbit IgG), conjugated with a fluorescent label, was used to incubate the slides for 1 hour (1:100 diluted in PBS) at room temperature. After rinsing with PBS the slides were then embedded with TRIS-glycerol (pH 8.0) containing 0.5 µg mL⁻¹ 4', 6-diamidin-2-phenylindol-dihydrochloride (DAPI; Sigma-Aldrich Chemie BV).

Lamin A and Lamin C could be detected simultaneously using a mix of 133A2 (mouse monoclonal antibody) and RaLC (affinity purified rabbit antiserum) for the incubation. The mix of secondary antibodies consisted of fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse (DAKO A/S) and a Texas Red-labeled goat-anti-rabbit antibody (SBA). Lamin A and Ki-67 were detected simultaneously using a mix of 133A2 and the rabbit polyclonal serum A0047.

Table 2: Major lamin expression patterns in normal skin, actinic keratosis (AK), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC)

Tissue	Lamin A	Lamin C	Lamin B1	Lamin B2
Normal skin (n = 5)				
basal layer	-	+/-		+
suprabasal layers	+	+		+
AK (n = 5)				
basal layer	++	+	(nucleoli/lamina)	+
suprabasal layers	+	+	(nucleoli)	+/-
SCC (n = 10)				
Epidermis overlying SCC				
basal layer	++	+		+/-
suprabasal layers	+	+		+/-
BCC (n = 15)				
Epidermis overlying BCC				
basal layer	++	+	(nucleoli/lamina)	+
suprabasal layer	+	+	(nucleoli/lamina)	+

- no staining, +/- staining in small fraction of the cells, + positive staining in most cells, ++ positive staining in all cells

Results

Table 2 shows the major lamin expression patterns in normal skin, AK, SCC and BCC.

Lamin expression in normal skin and actinic keratosis

Lamin A was found predominantly in the suprabasal cell layers of the normal epidermis (Fig. 1A), while lamin C was occasionally present also in the basal cell (Fig. 1B). Stromal cells stained positive for both lamins A and C. In AK, lamin A was expressed in all cells of the basal layer as well as in most cells of suprabasal layers in all cases (Fig. 1C). Lamin C was found in the most basal and suprabasal cell layers in all cases, showing a predominant nucleolar localization (Fig. 1D).

B-type lamins were found to be expressed throughout the entire epidermis (Fig. 2A,B), which was similar to the pattern seen in AK. Stromal cells only seldomly stained positive for lamin B1, whereas lamin B2 was present in these cells.

Lamin expression in squamous cell carcinoma

Lamin A was detected in all or most cells in the basal and suprabasal cell layers of the apparently normal epidermis overlying SCC, whereas lamin C was expressed in all or most basal cells and in all cells in the suprabasal cell layers of the apparently epidermis overlying SCC (Fig. 3A,B). The B-type lamins showed slightly reduced expression levels in the basal cell layer of the epidermis overlying SCC as compared to normal skin.

Lamin A was detected in all or most tumor cells, with lamin C being expressed in most tumor cells of all cases, both showing a nuclear lamina staining (Fig. 3C,D). B-type lamins were found in most tumor cells.

Lamin expression in basal cell carcinoma

In the apparently normal epidermis overlying BCC, lamin A (Fig. 4A) could be detected in all or most cells in the basal cell layer. Only one case showed expression in a small fraction of the basal cell layer. Most cells in the suprabasal layers stained positive. For lamin C (Fig. 4B), most cases stained positive in all or most basal cells while four cases showed staining in a small fraction of the basal cells. Most or a small fraction of cells stained positive in the suprabasal layers. The staining was predominantly present at the nucleoli. Lamin B1 showed positivity in all or most cells in the basal cell layer, while a small fraction of cells in the suprabasal layers stained positive. Lamin B2 was detected in all or most cells in the basal and suprabasal cell layers.

In BCC, lamin A stained positive in all or most tumor cells, showing a nuclear envelope localization (Fig. 4C). Lamin C stained positive in all or most tumor cells. Only one case expressed lamin C in a small fraction of tumor cells. The staining was predominantly localized in the nucleoli (Fig. 4D), while in two cases the nuclear envelope was also stained. Lamin B1 expression was detected in all or most tumor cells, only one case showing staining in a small fraction of tumor cells. Lamin B2 stained positive in most tumor cells.

Ki-67/lamin A co-expression

Ki-67 positivity in normal human epidermis was mainly restricted to the suprabasal cell layers of the epidermis; these cells were either scattered throughout this layer, or clustered in certain areas, with an average of 11% positive cells (Fig. 5A).

In BCC, Ki-67 stained positive in the tumor cells at the periphery of most of the nodules in the nodular type, and in a somewhat more variable pattern in the morpheaform type. However, areas with a high number of cells expressing Ki-67 occurred adjacent to areas with a lower number of Ki-67 positive cells. The average of Ki-67 expressing tumor cells in BCC was 45%. The epidermis overlying BCC also showed increased numbers of cells positive for Ki-67 (35%) as compared to the epidermis in normal human skin with suprabasal layers of the epidermis also showing positive cells (Fig. 5B). In SCC, Ki-67 expression was predominantly located at the periphery of the nodules with an average of 20% of tumor cells being positive. The epidermis overlying SCC showed an average of 18% of cells expressing Ki-67. AK showed an average of 31% of Ki-67 expressing cells in the epidermis.

Co-expression of lamin A and Ki-67 occurred in 62% of the proliferating (Ki-67 positive) tumor cells in BCC (Fig. 5C) and in 56% of the tumor cells in SCC.

Discussion

Previous studies have shown that the nuclear lamina participates in interphase chromatin organization, DNA replication and gene expression⁹⁻¹². Its integrity seems instrumental in the regulation of apoptosis²⁰. Therefore, it is possible that changes in the lamina can affect the balance between cell proliferation and apoptosis in various cell types. In this study we investigated the differential expression patterns of the nuclear lamins in normal human skin, AK and in non-melanoma skin cancers SCC and BCC.

The lamin A expression in the basal cell layer of the epidermis overlying BCC, SCC and AK, in contrast with its exclusively suprabasal localization in normal skin, may suggest an involvement of these basal cells in the carcinogenic process. Also, it cannot be excluded that factors originating from the tumors may induce this differentiated-like phenotype of the basal cells. Furthermore, based on this abnormal expression pattern, antibodies against lamin A may be used to differentiate between normal human skin and diseased skin. With respect to lamin A expression in BCC, a striking difference was observed when comparing our results with these of Venables *et al.*¹⁷. Although similar distribution patterns were obtained in normal human skin, and a partially overlapping panel of antibodies was applied, we found a higher frequency of lamin A expressing cells in BCC.

Figure 1: A-type lamins in normal human skin and actinic keratosis

A. Lamin A (133A2); predominantly found in the suprabasal cell layers of the epidermis of normal human skin.

B. Lamin C (RaLC); occasionally present in basal cells next to a more homogeneous expression in the suprabasal layers of the epidermis of normal human skin.

C. Lamin A (133A2); expressed in the basal cell layer as well as in the majority of the cells in the suprabasal cell layers in actinic keratosis.

D. Lamin C (RaLC) showing a nucleolar staining pattern in the

basal, as well as in the suprabasal cell layers.

Original magnification used for photography of images A-D was 20x.

Figure 2: B-type lamins in normal human skin.

Lamin B1 (119D5F1, A) and Lamin B2 (LN43, B) are both expressed throughout the entire epidermis of normal human skin.

Original magnification used for photography of images A and B was 20x.

Figure 3: Lamin expression in squamous cell carcinoma and in the epidermis overlying squamous cell carcinoma

A: Lamin A (133A2) showing a homogeneous staining of the basal cells of the epidermis overlying squamous cell carcinoma, the majority of the suprabasal cells also staining positive.

B: Lamin C (RaLC); also expressed in the majority of cells of the epidermis overlying squamous cell carcinoma, with a localization at the nuclear envelope.

C: Lamin A (133A2);

homogeneously expressed at the nuclear lamina in the tumor cells.

D: Lamin C (RaLC); expressed in the majority of tumor cells and also showing a localization at the nuclear envelope.

Original magnification used for photography of images A-D was 20x.

Figure 4: Lamin expression in basal cell carcinoma and the epidermis overlying basal cell carcinoma
 A: Lamin A (133A2); homogeneously expressed in the basal cell layer of the epidermis overlying basal cell carcinoma and the suprabasal cells also showing expression in the majority of the cells.
 B: Lamin C (RaLC); expressed in the majority of the cells of the epidermis overlying basal cell carcinoma, and showing localization at the

nuclear envelope next to localization at the nucleoli.

C: Lamin A (133A2); homogeneously expressed in the tumor cells.

D: Lamin C (RaLC); expressed in the majority of tumor cells but showing a localization at the nucleoli.

Original magnification used for photography of images A-D was 40x.

Figure 5: Ki-67/lamin A co-expression in normal human skin and basal cell carcinoma (BCC)

A: Lamin A (green) and Ki-67 (red) in normal human skin: Ki-67 positivity is mainly restricted to the suprabasal cell layers as is the expression of lamin A.

B: Lamin A (green) and Ki-67 (red) in the epidermis overlying basal cell carcinoma: Ki-67 positivity is enhanced as compared to that in normal human epidermis. Lamin A is homogeneously expressed in the basal cell layer of the epidermis overlying basal cell carcinoma in contrast to its absence in basal cells of normal human skin.

C: Co-expression of lamin A (green) and Ki-67 (red) in basal cell carcinoma.

Original magnification used for photography of images A and B was 40x, for image C 20x.

The mechanism by which the aberrant localization of lamin C in AK and BCC occurs is still unclear. Nuclear foci of lamin expression have been reported before in the case of A-type lamins^{25,26}. The authors suggested that these clustered A-type lamins form deposits from which the lamins will finally be transported to and assembled in the peripheral nuclear lamina. Aberrant expression of A-type lamins has furthermore been reported in non-small cell lung carcinoma¹⁶. Cytoplasmic localization of lamin A was observed and partially explained by the possibility that the lamins were improperly assembled or even truncated. Deletion of either the lamin A N-terminal domain or the C-terminal CaaX domain results in the formation of intra-nuclear aggregates and disruption of the endogenous lamins A and C²⁷. Vaughan *et al.*²⁸ showed that absence of lamin A from the nuclear envelope correlated with a significant fraction of lamin C being mis-localized to the nucleolus. Although it thus seemed likely that lamin A is essential for the localization of lamin C to the nuclear envelope, the present study shows that the mis-localization of lamin C to the nucleolus appears to be independent of lamin A. Jagatheesan *et al.*²⁹ found a speckled pattern of expression for A-type lamins in HeLa cells. These speckles were found to co-localize with RNA splicing factors and the speckles dispersed when the cells entered mitosis. The speckled pattern of expression was also seen by Hozak *et al.*³⁰, who stated that internal lamins are normally buried in dense chromatin and are thus inaccessible for antibodies. After removal of the chromatin, the internal 'speckly' labeling increases for both lamin A and lamin A/C antibodies. However, no effect was seen on the labeling of lamin B2, which remained predominantly located at the peripheral lamina. Furthermore, lamin foci were found to be associated with sites of DNA replication³¹. In addition, mis-localization of lamin C to the nucleolus might be a first step towards its absence in higher-grade tumors as is suggested in the studies of Venables *et al.*¹⁷. It is at least remarkable that the nucleolar localization is restricted to AK and BCC, and not seen in SCC and normal epidermis.

Regarding simultaneous expression of lamin A and Ki-67, our findings corroborate with previous studies stating that AK possesses more proliferative capacity than SCC³²⁻³⁵. The differences in proliferation may partially explain their differential clinical behavior.

In normal tissue A-type lamins are in general restricted to differentiated, non-proliferative, cells⁵. In the skin tumors examined, however, we found co-expression of lamin A and Ki-67 in approximately 60% of the proliferating cells. This simultaneous expression was also observed by Janssen *et al.*¹⁴ who showed that a considerable fraction of the Ki-67 positive Reed-Sternberg and Hodgkin cells expressed also A-type lamins, indicating that those proliferating cells had a differentiated phenotype. Our recent results in BCC are contrasted by the findings of Venables *et al.*¹⁷, who reported mutually exclusive staining patterns for lamin A and Ki-67. The major methodological differences between the two studies were that Venables *et al.*¹⁷ used single staining of serial sections and therefore were unable to demonstrate conclusively that lamin A was absent from Ki67 positive cells. Furthermore, there seemed to be an apparent correlation between the absence of lamin C and a low proliferative index in their series of BCC. This conclusion was also based on staining of serial sections with the individual antibodies. We feel that the double-staining

protocol used in the present study may allow a more accurate comparison of the distribution patterns of Ki-67 and lamin A. We therefore conclude that the double-stained malignant cells are of a differentiated phenotype but still possess proliferative capacity.

A sequence of change in lamin expression may characterize basal cell skin carcinoma. Based upon the findings reported here and those reported previously¹⁷, four stages in the development of BCC might be revealed by different patterns on A-type lamin expression. Venables *et al.*¹⁷ reported a high incidence of lamin A negative, Ki-67 positive tumors (stage 1) and suggested that this group was representative of the origins of the tumor since basal cells tend to be lamin A negative and Ki-67 positive in normal skin. In this study a significant group of BCCs were lamin A positive and Ki-67 positive (stage 2). Up-regulation of lamin A expression in this instance might accompany tumor de-differentiation. In these tumors a fraction of lamin C was located in the nucleolus. In Venables studies¹⁷, lamin C was found in the nucleolus in one tumor (stage 3) and was completely absent (stage 4) in a number of others. All of these tumors had a low proliferative index. Since BCC is characterized by slow growth and an absence of metastasis, this might represent a final phase in the differentiation process. Thus we propose that tumor progression in BCC is characterized by absence of lamin A at its origin (stage 1), but as the tumor progresses lamin A expression is up-regulated (stage 2). During these two phases tumor cells divide rapidly. As growth slows down, lamin C is first re-located to the nucleolus (stage 3) and subsequently its expression is largely diminished (stage 4). Whether these changes in lamin expression influence or reflect changes in the growth of the tumor is yet to be investigated.

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Chapter 5: Therapy of BCC

The garlic derived organosulfur component ajoene decreases basal cell carcinoma tumor size by inducing apoptosis

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Abstract

Although the therapeutical role of ajoene, an organosulfur compound of garlic, in cardiovascular diseases and mycology has been established, its usefulness in cancer treatment has only recently been suggested. We have applied ajoene topically to 21 patients with either nodular or superficial basal cell carcinoma (BCC), resulting in a reduction of tumor size in seventeen patients. Immunohistochemical assays for Bcl-2 expression in a selection of these tumors before and after treatment showed a significant decrease of this apoptosis-suppressing protein. On average, the percentage of tumor cells expressing the proliferation marker Ki-67 was not decreased, which contradicts a cytostatic action of ajoene. To obtain further insight into the mode of action of ajoene, BCC cell line TE354T and short-term primary culture of BCC were analyzed for apoptosis induction after treatment with the drug. Apoptosis was detected by morphology of the cells and by flow cytometry. It could be demonstrated that ajoene induces apoptosis in a dose and time dependent manner in these cultures.

Taking together the *in vivo* and *in vitro* studies, we conclude that ajoene can reduce BCC tumor size, mainly by inducing the mitochondrion-dependent route of apoptosis.

Keywords: ajoene, apoptosis, basal cell carcinoma, Bcl-2, cell cycle

Introduction

Basal cell carcinoma (BCC), a non-melanoma type of skin cancer, is the most common malignancy in Caucasians. Although generally of indolent nature, occasionally the tumor can become very aggressive and destructive¹. For treatment of BCC several surgical and non-surgical therapies are available². Standard therapies include primary surgical excision, Mohs' micrographic surgery and cryosurgery. Surgical therapy is preferred in the case of uncomplicated BCCs³. The medical history of the patient, age, tumor localization and size, physical condition and histological outcome will eventually determine the therapy choice. Non-surgical treatment schedules become increasingly important because of better cosmetic result. Non-invasive therapies like photodynamic therapy (PDT) that uses 5-aminolevulinic acid (5-ALA) as a photosensitizer^{4,5} are still under investigation. Topical application 5-fluorouracil has also been proven successful in the treatment of BCC⁶.

One of the approaches by which BCC can be treated is by the induction of apoptosis in the tumor cells. Our earlier studies do however indicate that a major fraction of BCC is largely positive for the anti-apoptotic Bcl-2^{7,8}, thus suggesting a protection of the tumor cells against programmed cell death. Drugs that can induce this process in BCC may therefore become important tools in non-surgical therapy of this widespread disease.

More and more phytochemicals are being applied in cancer prevention and therapy⁹. Several epidemiological studies have revealed that garlic consumption has played a significant role in the reduction of deaths caused by malignant diseases. The mortality rate among patients with gastric cancer in China was significantly lower in patients with higher garlic consumption (about 20g/day) than in patients with lower garlic consumption¹⁰. Similar effects were seen in gastric cancer patients in Italy¹¹. Since then, a number of studies have appeared on the anti-tumor and cytotoxic actions of garlic and its organosulfur compounds.

Ajoene, an organosulfur compound of garlic, possesses anti-fungal activity¹², inhibits platelet aggregation¹³, and has been shown to induce apoptosis in human promyeloleukemic cells¹⁴. Furthermore, these authors showed that ajoene can inhibit the expression of inducible nitric oxide synthase (iNOS), a major enzyme in the production of nitric oxide (NO) in activated macrophages¹⁵. During inflammatory processes cytokines induce the expression of iNOS in endothelial cells, followed by high output NO synthesis^{16,17}. This NO is thought to serve as nonspecific immunoprotector¹⁸ and can mediate the onset of apoptosis in a variety of mammalian cells¹⁹. Suschek et al.²⁰ reported that NO prevents UV-induced apoptosis in the skin and that this NO-mediated protection tightly correlates with over-expression of the apoptosis-inhibiting component Bcl-2. Therefore, we studied the expression patterns of the Bcl-2 protein before and after ajoene treatment of BCC to determine its apoptosis inducing effect.

Since several authors^{21,22} suggested an effect of ajoene on cell cycle progression, we furthermore investigated the effect of ajoene on proliferative activity of the tumor cells by determining Ki-67 expression.

Materials and Methods

Ajoene

Ajoene [(E)-4,5,9-trithiododeca-1,6,11-triene-9-oxide] was purified from alcoholic extracts of garlic as described before [23]. Briefly, allicin (a diallyl thiosulfinate that is produced when garlic is crushed) produces ajoene by incubation of a 2% solution of allicin in acetone/water (3/2) at 80°C for 10h. The crude product is then purified by column chromatography.

Ajoene treatment in vivo

Twenty-one patients (12 males and 9 females, in age ranging from 49-91 years) with twenty-five individual histologically proven BCC of nodular ($n = 17$) or superficial ($n = 8$) type with a maximum diameter of 2 cm were selected. Patients with a suppressed immune system or using immune-suppressive drugs were excluded. All patients signed an informed consent prior to their inclusion in the study, which was approved by an Institutional Review Board (Ethics Committee). Before treatment, the surface area (mm^2) of the lesions was determined and a biopsy (3mm diameter) of the tumor was taken, fixed in 4% formaldehyde and embedded in paraffin. The BCCs were then treated by local application twice a day of a 0.4% ajoene cream (400 mg Ajoene, 0.3 ml polysorbate 80, and 0.3 ml sorbitonolate in 100 ml 1 % carbomeric gel) extending 1 cm beyond the tumor margins. The BCCs were then covered with a hydrocolloid dressing. The lesions were measured once a month. After treatment for 6 months, the BCCs were completely removed by surgical excision, fixed in 4% formaldehyde and embedded in paraffin.

Immunohistochemistry of tissue sections

Paraffin sections (5 μm) were cut onto Superfrost plus slides (MERCK, Darmstadt, Germany), dried overnight at 56°C, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 /methanol and a microwave antigen-retrieval step was performed. The sections were then incubated with the primary antibodies Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or MIB-1 (Immunotech, Marseille, France) both diluted 1:50 in PBS, followed by incubation with the appropriate secondary antibodies, and detection via peroxidase with the ABC complex (Vector Laboratories Inc., Burlingame, CA, USA). Peroxidase activity was detected using 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) as chromogen and the sections were counterstained using haematoxylin (Fluka AG, Buchs, Germany). After dehydration, the sections were then embedded using Entellan (MERCK).

The percentage of tumor cells expressing Ki-67 and Bcl-2 was established by counting a minimum of 500 cells per slide.

BCC cell cultures

The human BCC cell line TE354T (ATCC, Rockville, MD, USA) was cultured in DMEM (ICN Biomedicals, Cleveland, OH, USA) containing 10% FCS, 1% L-glutamine and 0.1% gentamycin.

For primary cultures, BCC tumor tissue was obtained after informed consent, from patients undergoing Mohs' micrographic surgery, cut into small pieces and rinsed in calcium- and magnesium-free PBS. Then the tumor pieces were incubated overnight in DMEM containing 0.125% trypsin and 1% gentamycin at 37°C. After incubation, the trypsinization was blocked by adding an equivalent amount of fetal calf serum (FCS) and by centrifugation of the solution for 5 minutes at 1200 rpm. The pellet was resuspended in serum-free keratinocyte medium (GIBCO, Paisley, Scotland, UK) and the washing step was repeated several times. Then the cells were inoculated in a small flask and put back at 37°C. Medium was refreshed every other day. Fibroblasts were removed at sub-confluency by adding a solution of 0.125% trypsin and 0.02% EDTA in PBS for 30-60 seconds at room temperature. Loosened fibroblasts could then be decanted and the remaining cells were washed several times with medium. Normal keratinocytes are removed by increasing the calcium concentration from 0.09 mM to 1.6 mM for a period of 5-7 days, after which the normal keratinocytes should be differentiated and non-proliferative. The remaining BCC colonies can then be returned to low calcium concentration medium.

Immunohistochemistry of cell cultures

The cell lines were characterized by immunohistochemical detection of several cytokeratins (CK). CK5 and CK14, detected by mouse monoclonal antibodies RCK102²⁴ and RCK107^{25,26}, respectively, are the basal cell keratins in combined and stratified epithelial tissues. CK8, CK18 and CK19 are present in glandular epithelial structures and carcinomas originating from them, and are detected by mouse monoclonal antibodies M20²⁷, RCK106²⁸ and RCK108²⁹ respectively. Briefly, the cells were fixed on microscopy cover slides, rinsed in PBS and incubated overnight with the primary antibody at 4°C. After rinsing with PBS, the cells were then incubated with the appropriate fluorescent-labeled secondary antibody at room temperature for 1 hour. After another washing step with PBS, the slides are mounted in TRIS-glycerol (pH 8.0) containing 0.5 µg/mL 4', 6-diamidin-2-phenylindol-dihydrochloride (DAPI; Sigma-Aldrich Chemie BV).

Ajoene treatment of cell cultures

Ajoene was dissolved in dimethylsulfoxide (DMSO; MERCK) and further diluted in the appropriate medium. Ajoene was added to the cells for increasing time periods (6-24 hours) and in three different concentrations i.e. 1 mM, 5 mM and 10 mM. Final DMSO concentration was less than 0.1% and has been shown not to interfere with the test results in control studies.

Apoptosis assay

The number of apoptotic cells was quantified using the annexin-V assay³⁰. After incubation with ajoene the cells are rinsed several times in PBS and once in 1ml annexin-binding buffer. The pellet was then resuspended in a solution containing annexin-V-FITC (1:500) (Nexins Research BV, Hoeven, The Netherlands) and propidium iodide (PI). Multi-parameter flow cytometry analysis was used to assess ajoene-induced apoptosis. In early stages of apoptosis, phosphatidylserine is exposed at the external surface of the cell and can be

detected by annexin-V. Late apoptotic cells and necrotic cells will also show PI positivity. Living cells, however will show neither annexin-V, nor PI positivity.

Flow cytometry

Flow cytometric analyses were performed using the FACSort flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with a single Argon ion laser. Fluorochromes were excited at 488nm, and the emission filters used were 515-545 BP (green, FITC) and 600 LP (red, PI). A minimum of 10^4 cells per sample was analyzed and data stored in list mode. FITC signals were recorded as logarithmic amplified data, while the PI signals (DNA content per cell) were recorded as linear amplified data. For bi-variate FITC/PI analysis no compensation was used. Forward and side scatter of cells was recovered to observe morphological changes of cells. All analyses were performed with Cell Quest software (Becton Dickinson).

Figure 1: Effect of ajoene on BCC tumor size after topical application in patients.

BCC located on the forehead (A,B) and on the abdomen (C,D), before (A,C) and after (B,D) topical treatment with ajoene. Tumor surface areas are as follows A: 63mm^2 , B: 42mm^2 , C: 195mm^2 , D: 24mm^2 .

Results

To study the effect of ajoene on BCC, 21 patients with in total 25 lesions were treated by exogenous application of the organosulfur compound. The in vivo results were verified and extended by in vitro studies on human BCC cell lines.

Effect of ajoene on tumor size, Bcl-2 and Ki-67 expression

Although the patient studies showed heterogeneous results, and immunohistochemistry indicates that only a subset of the cells is targeted, the abundance of BCCs showed a positive response to ajoene with respect to size reduction and decrease of Bcl-2 expression.

Figure 1 shows the effect of ajoene containing cream on the surface area of BCCs of two patients after treatment during 6 months. Of the cases examined, 3 tumors showed an increase in tumor size, in 1 tumor no change in size was noted while in 21 samples tumor size was reduced (Fig. 2). On average, the size of the BCCs in this study showed a reduction with a median value of 47% (ranging from 69% increase to 88% decrease) ($p < 0.01$; Wilcoxon), of which 42% (ranging from 69% increase to 82% decrease) for the nodular type (case numbers 1, 4-7, 9-15, 17, 19, 21, 23 and 24) and 55% (ranging from 19% increase to 88% decrease) for the superficial type of BCC (case numbers 2, 3, 8, 16, 18, 20, 22 and 25).

Figure 2: Effect of ajoene on tumor size after topical application in the individual samples.

Samples were aligned as follows: 1-3 Tumors of increased size after treatment; 4 Tumor without change in size; 5-25 Tumors of decreased size after treatment.

Figure 3A shows a selection of 10 cases covering tumors that show either growth, standstill or regression under influence of ajoene. Figure 3B shows the percentage of Bcl-2 expressing tumor cells per tumor before and after treatment with ajoene in this selection. It is obvious that in all cases but one the number of cells expressing Bcl-2 decreased dramatically i.e. a median reduction value of 47% (ranging from 4% increase to 72% decrease) ($p < 0.01$; Wilcoxon). For the nodular type BCC (sample numbers 1, 4, 5, 12, 17 and 21) this approximated 39% (ranging from 4% increase to 72% decrease) and for the superficial type BCC (sample numbers 2, 3, 22 and 25) 50% (ranging from 33% decrease to 69% decrease).

Figure 3C shows the percentage of Ki-67 expressing tumor cells per sample before and after treatment. It can be seen that in six cases a decrease in proliferation index is detected, while in four cases the number of Ki-67 positive cells was increased. When nodular and superficial type of BCC were analyzed separately, nodular BCC (sample numbers 1, 4, 5, 12, 17 and 21) showed a

decrease with the median value of 2% (ranging from 167% increase to 25% decrease) of Ki-67 positive cells, in contrast to a decrease for the superficial type of 21% (ranging from 10% increase to 72% decrease) (sample numbers 2, 3, 22 and 25). On average, no significant change was detected for the percentage of Ki-67 expressing cells before and after ajoene treatment. In fact, four of the ten (40%) samples examined show an increase of the number of Ki-67 positive cells after ajoene treatment. This indicates that the effect of ajoene on cell proliferation is not consistent in BCC.

Figure 3: Correlation of the effect of ajoene on tumor size, Bcl-2 and Ki-67 expression in a selection of BCC. Samples were selected based on their divergent response to ajoene, i.e. 1-3 Tumors of increased size after treatment; 4 Tumor without change in size; remaining cases show tumors of decreased size after treatment. A: BCC surface area (mm²) before and after ajoene treatment. B: Percentages of Bcl-2 expression in BCC tumor cells before and after ajoene treatment. C: Percentages of Ki-67 expression in BCC tumor cells before and after ajoene treatment.

Figure 4: Bcl-2 (A,B) and Ki-67 (C,D) staining in BCC before (A,C) and after (B,D) ajoene treatment.

Original magnifications for images A-B: 40x, for images C-D: 20x

Figure 4 shows examples of immunohistochemical staining patterns for Bcl-2 (Fig. 4A, B) and Ki-67 (Fig. 4C, D) in BCC, either before (A,C) or after (B,D) treatment for 6 months with ajoene. Bcl-2 showed a homogeneous staining pattern before ajoene treatment in contrast to a clearly reduced expression pattern after topical application of ajoene. For Ki-67, no significant changes in the number of expressing cells could be detected.

CK expression profile of BCC cell lines

Cells of both TE354T and the short-term BCC cell culture showed strong positive immunoreactivity to the antibodies directed against CK 5 and 14 in the majority of the cells (results not shown), whereas no reactivity could be detected for CK18. Positivity for CK8 and CK19 was found in a small fraction of the cells. This CK-profile is compatible with a BCC origin of the cell cultures 31,32.

Effect of ajoene on apoptosis in BCC cell cultures

To further investigate the underlying mechanism of the (potentially) growth inhibitory effect of ajoene *in vivo*, we used BCC cell cultures for more advanced apoptosis assays. The results of the annexin-V assay are depicted in Figure 5. It can clearly be seen that the apoptosis induction in BCC cell culture, either commercially available or derived from short-term cell culture, is both dose- and time-dependent. It can be noted that BCC cells from short-term culture are more susceptible for apoptosis induction by Ajoene than the commercially available BCC cell line, a phenomenon that may be related to a higher growth rate noted for the short-term culture. Furthermore, 10mM Ajoene seems to have an almost optimal effect on apoptosis induction, especially after 24 hours.

Figure 5: The effect of incubation with ajoene on cell viability in TE354T cell line (A-C) and short-term culture of basal cell carcinoma (D-F). The fraction of living cells is depicted as squares, the fraction of apoptotic cells is depicted as dots.

Discussion

Several therapies for the treatment of BCC exist². In general, surgical excision is the therapy of first choice, but it may be obvious that a non-invasive treatment is preferred. Here we show for the first time that ajoene, a sulfur-containing compound of garlic, is effective in the treatment of BCC. The present study also aimed at elucidating the underlying biological effect of this phytochemical. We showed that 84% of the tumors, treated with an ajoene-containing cream were significantly reduced in size after 6 months of treatment. It was shown that the superficial tumors showed a larger reduction in size next to a larger reduction in the number of cells expressing Bcl-2, as compared to the nodular tumors, which may be explained by the fact that the ajoene cream can more easily penetrate the superficial tumors. A similar differential effect on tumor size reduction was previously seen in case of treatment of BCC with PDT. Complete initial clearance of more than 90% for superficial lesions was seen in contrast to 10-64% for nodular BCC^{33,34}. Another possible topical treatment for BCC is imiquimod. Imiquimod is an immune response modifier that has been demonstrated to induce cytokines such as interferon-alpha, interferon-gamma and interleukin 12 that promote an immune response in the tumor cells^{35,36}. However, in the majority (92%) of BCC patients treated with imiquimod, local reactions were seen at the tumor site including erythema, itching, crusting and even severe erosion³⁷. This is in contrast to our study using ajoene where no significant side effects were observed.

When comparing the Ki-67 immunostaining results before and after topical application of ajoene to BCC in patients, no reproducible shift in the percentage of Ki-67 positive cells was noted. BCCs showing an increased proliferative cell fraction were seen next to tumors with a decreased proliferative activity. Therefore we can conclude that in BCC patients the anti-tumorigenic effect of ajoene is not only explained by a cytostatic effect of the drug.

Tumor size reduction may therefore be better explained by the apoptosis-inducing ability of ajoene. To test this hypothesis we have applied an assay to directly prove a diminished protection against apoptosis, rather than procedures to detect apoptotic cells in BCC, such as the TUNEL assay³⁸. The latter protocol normally also detects necrotic cells and is therefore non-specific, which can explain the divergent results in the literature, showing between 0.6% and 51% of apparently apoptotic cells in BCC³⁹⁻⁴¹. The Annexin V-assay³⁰ or the M30 Cytodeath assay⁴² cannot be used in case of tissue sections of BCC. Furthermore, the real apoptotic fraction is rapidly cleared from the tumor by phagocytosis. Immunohistochemical staining of the tumors treated with ajoene revealed a decreased number of cells expressing Bcl-2. This provides a possible mechanism for the effect of ajoene on BCC. By reducing the number of Bcl-2 positive, apoptosis-resistant, tumor cells, ajoene may increase the susceptibility for apoptosis in the tumor cells, which will eventually result in a reduction of tumor size.

This finding is furthermore supported by the observation that, when added to the culture medium of BCC cells, ajoene induces apoptosis in these cells. This apoptosis-inducing effect is both time and dose dependent. Taken together,

these findings provide an important step in dissolving the underlying molecular mechanisms of the anti-tumor action of ajoene.

Recently Dirsch et al.⁴³ and Ahmed et al.⁴⁴ proved that ajoene induces apoptosis in leukemic cell lines by triggering the mitochondria-dependent caspase cascade. The authors furthermore reported that Bcl-2 expression was significantly decreased in these cells⁴⁴ and that over-expression of Bcl-x(L), an apoptosis-regulating protein, clearly diminished the ajoene-induced caspase-activation as well as apoptosis⁴³. Kwon et al.⁴⁵ reported that diallyl disulfide (DADS), another component of garlic, induces apoptosis of human leukemia HL-60 cells in a concentration- and time-dependent manner.

We conclude that ajoene can have a diminishing effect on the growth of BCC by influencing the disturbed balance between proliferation and apoptosis. Our earlier studies showed that on average 6% of the tumor cells in BCC show both an increased proliferative activity as well as a reduced apoptotic susceptibility⁷. It is most likely that this progressive cell population is the most sensitive target for ajoene treatment.

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Summary

Basal Cell Carcinoma (BCC) is a non-melanoma type of skin cancer that accounts for almost 80% of all skin malignancies and is the most common malignancy among Caucasians. Since apoptosis and proliferation are two important processes to be considered when studying growth characteristics of tumor, this thesis mainly concentrates on the (dis)balance between these two phenomena in BCC.

In the **first chapter** the molecular cell biology of BCC is being reviewed. Clinical features and the risk factors are discussed. Next to the obvious environmental risk factors such as exposure to sunlight and toxic agents, there are a number of genetically determined factors. Several syndromes exist, like Xeroderma Pigmentosum and Nevoid Basal Cell Carcinoma Syndrome, that may lead to a higher susceptibility for the development of BCC. Also immuno-suppression seems to play a major part in predisposing for the development of skin cancer. A subject of major interest is the origin of the tumor. There are several arguments for an inter-follicular epidermal origin, but most evidence points towards an origin from the hair follicle, and more specifically, the stem cells of the follicle, located in the bulge area.

Therefore, in the **second chapter** the human hair follicle is studied immunohistochemically, using the stem cell marker cytokeratin 19 and the apoptosis-suppressing protein Bcl-2, to detect the localization of the stem cells. It seems that indeed two areas exist that may provide these cells. When the BCCs are studied for the expression pattern of Bcl-2 (**chapter 3.1**), we see that the tumors express this apoptosis-regulating protein homogeneously. Remarkably however, the apparently normal epidermis overlying BCC also shows an enhanced expression of Bcl-2, in contrast with a strict basal cell localization in the healthy epidermis. This suggests that superficial tumors may arise from the basal cells, whereas the nodular and morphea type may originate from the hair follicles, and may explain why both epidermis and hair follicle show comparable patterns of expression for some proteins such as cytokeratins and cell adhesion molecules. This is also found in the study using other Bcl-2 like proteins (**chapter 3.2**), where BCCs show a basal cell-like phenotype and the overlying epidermis again shows an intermediate expression pattern between normal epidermis and BCC. Whether this phenomenon is induced by factors originating from BCC or can be regarded as a first step in skin carcinogenesis remains to be established.

In **chapter 4** the differential expression patterns of nuclear lamins, which are related to differentiation and proliferation, are shown in different stages of skin cancer. Significant changes occur in the expression patterns of A-type lamins in both pre-malignant and malignant lesions of the skin. The profound overlap of staining patterns of lamin A and the proliferation marker Ki-67 indicates that the proliferating tumor cells may obtain a certain degree of differentiation. Also, lamin A expression in the basal cell layer of the apparently normal epidermis

overlying BCC again suggests its involvement in the primary process. Furthermore, a sequence of four stages in the development of BCC is revealed by different patterns of A-type lamin expression. Stage 1 is represented by a high incidence of lamin A negative, Ki-67 positive tumors. As the tumor progresses to stage 2, lamin A expression is upregulated. During these two first stages the tumor shows a high degree of proliferation. When growth slows down in stage 3, lamin C expression is first relocated to the nucleoli and subsequently its expression is largely diminished during stage 4, the stage at which the cells obtain their final BCC phenotype.

In **chapter 5** a possible new treatment for BCC is introduced by applying ajoene, an organosulfur compound of garlic, directly to the tumors. The *in vivo* studies showed a clear diminishing effect on tumor size in most cases and suggested selective induction of apoptosis by down-regulating the expression of the anti-apoptotic Bcl-2 protein. These findings were furthermore supported by *in vitro* studies, showing that ajoene can induce apoptosis in both a BCC cell line and a short-term primary culture of BCC. A major advantage of this treatment is that ajoene showed no side effects in normal skin. This study provides a good basis to further investigate apoptosis-inducing agents for their future application in BCC treatment.

Samenvatting

Het basaalcelcarcinoom (BCC) is een niet-melanoma type huidkanker dat ongeveer 80% van alle maligne huidaandoeningen uitmaakt. Het is de meest voorkomende maligniteit in mensen van het Caucasische ras. Aangezien apoptose en proliferatie twee belangrijke processen zijn die in acht genomen moeten worden wanneer men groeikarakteristieken van een tumor onderzoekt, concentreert deze thesis zich met name op de (verstoorde) balans tussen deze twee fenomenen in BCC.

In **Hoofdstuk 1** werd de moleculaire celbiologie van BCC besproken. Klinische kenmerken en risico-factoren worden besproken. Naast de overduidelijke omgevingsfactoren zoals blootstelling aan zonlicht en gifstoffen, zijn er ook een aantal genetische factoren. Er bestaan verschillende syndromen, zoals bijvoorbeeld Xeroderma Pigmentosum en Nevroid Basaal Cel Carcinoma Syndroom, die aanleiding geven tot een hogere gevoeligheid voor de ontwikkeling van BCC. Daarnaast speelt immuunsuppressie ook een grote rol. Een onderwerp van grote belangstelling is het ontstaan van een BCC. Er zijn verschillende argumenten die duiden op een oorsprong in de interfolliculaire epidermis, echter de meeste bewijzen duiden op een oorsprong in de haarfollikels, en meer specifiek, de stamcellen van de follikel, gelocaliseerd in het zogenaamde 'bulge' gebied.

Daarom werd in **Hoofdstuk 2** de menselijke haarfollikel onderworpen aan een immunohistochemische analyse, gebruik makend van de stamcel merker cytokeratine 19 en het apoptose-onderdrukkende Bcl-2 eiwit, om de localisatie van de stamcellen te determineren. Er blijken inderdaad twee gebieden aanwezig te zijn die mogelijk stamcellen leveren. Wanneer BCCs immunohistochemisch bestudeerd worden voor de expressie van het Bcl-2 eiwit (**Hoofdstuk 3.1**), zien we dat de tumoren dit apoptose-regulerend eiwit homogeen tot expressie brengen. Opmerkelijk is echter, dat de schijnbaar normale epidermis boven de tumor ook een verhoogde expressie van het Bcl-2 eiwit vertoont, in tegenstelling tot wat we zien een gezonde huid, waar het Bcl-2 eiwit enkel in de basal laag tot expressie komt. Deze bevindingen suggereren dat de superfaciele tumoren ontstaan van de interfolliculaire epidermis, en dat de nodulaire en sprieterige types ontstaan uit de haarfollikels, en verklaren tevens waarom zowel epidermis als haarfollikels vergelijkbare expressie patronen van eiwitten zoals de cytokeratines en cel-adhesie moleculen vertonen. Dit werd ook geconstateerd in een studie naar Bcl-2-gerelateerde eiwitten in BCC (**Hoofdstuk 3.2**), waar BCCs een fenotype vertonen gelijkaardig aan de basale cellen, en de overliggende epidermis opnieuw een expressie patroon vertoont dat tussen dat van gezonde huid en BCC ligt. De vraag of dit fenomeen wordt geïnduceerd door factoren die afkomstig zijn van het BCC of dat dit gezien kan worden als een eerste stap in het ontstaan van huidkanker blijft voorlopig nog onbeantwoord.

In **Hoofdstuk 4** werden de differentiele expressie patronen van de nucleaire lamines, eiwitten gerelateerd aan proliferatie en differentiatie, besproken in de verschillende stadia van huidkanker. Significante veranderingen treden op in de expressie patronen van de A-type lamines in zowel pre-maligne als maligne huidaandoeningen. De overduidelijke simultane expressie van lamine A en de proliferatie merker Ki-67 duidt op het feit dat de prolifererende tumor cellen beschikken over een zekere graad van differentiatie. Verder duidt de aanwezigheid van lamine A in de basale laag van de schijnbaar normale overliggende epidermis op een betrokkenheid van deze cellaag in het primaire proces. Op basis van de differentiele expressie patronen van lamine A kunnen er vier stadia onderscheiden worden in de ontwikkeling van een BCC. Het eerste stadium wordt gekenmerkt door de aanwezigheid van lamine A negatieve, Ki-67 positieve tumoren. Progressie naar stadium 2 gaat gepaard met op-regulatie van de expressie van lamine A. Gedurende deze twee eerste stadia vertonen de tumoren een hoge proliferatie index. Wanneer de proliferatie afneemt in het derde stadium, wordt lamine C herlocaliseerd naar de nucleoli alvorens gedurende stadium 4 te verdwijnen; dit is het stadium waar de cellen hun uitendelijke BCC fenotype aannemen.

In **Hoofdstuk 5** werd een mogelijke nieuwe behandeling voor BCC geïntroduceert, door een directe applicatie van ajoene, een bestanddeel van knoflook. De in vivo studies vertonen een duidelijke reductie in tumor afmeting in het merendeel van de bestudeerde patiënten en suggereren een selectieve inductie van apoptose door vermindering van de expressie van het apoptose-onderdrukkende eiwit Bcl-2. De resultaten werden bevestigd door in vitro studies waaruit is gebleken dat ajoene apoptose kan induceren in zowel een korte-termijn kweek van BCC als een commercieel verkrijgbare BCC cellijn. Een groot voordeel van deze behandeling is dat ajoene geen bijwerkingen vertoont in de normale huid. De studie levert een goede basis voor de verder studie naar apoptose-inducerende reagentie en hun toekomstige toepassing in behandeling van BCC.

Dankwoord

'Hoe moet ik hier nu aan beginnen?', dat vroeg ik me af toen ik aan mijn dankwoord wilde beginnen. Het dankwoord, de tekst die als laatst geschreven wordt, maar waarschijnlijk als eerst gelezen. Bij de ontvangst van proefschriften zie je altijd iedereen direct doorbladeren naar de laatste bladzijden, om te kijken wie er al dan niet vermeld wordt in het dankwoord. Ik moet toegeven, dat deed ik ook altijd. Als tweede waren dan de stellingen aan de beurt. Eigenlijk is dat toch wel schandalig, de stukken die het minst met het onderzoek te maken hebben worden als eerste grondig bestudeerd en door sommigen hevig bekritiseerd. Wat weten zij daar nu van? Velen van hun weten niet welke zware taken het zijn om deze tekstjes te produceren. Waarschijnlijk beseffen ze niet dat hier meer bloed, zweet, tranen en slapeloze nachten aan vooraf zijn gegaan dan voor de rest van het 'boekje'. De (soms) prachtig geïllustreerde verhalen die het grootste deel van het boek uitmaken, zijn immers op voorhand al uitbundig besproken met de nodige co-auteurs en de AIO wordt als het ware aan het handje geleid naar het einde. Maar dan, het dankwoord, de grote test voor iedere schrijver, nu komt het enkel en alleen op de AIO zelf aan. En 'wee je gebeente' als je iemand vergeten bent.

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Bedankt!
Claudia

Curriculum Vitae

Claudia Tilli werd geboren op 27 mei 1976 te Maastricht. Na voltooiing van het Gymnasium aan het Trichter College te Maastricht in 1992, werd de studie Scheikunde aangevangen aan het Limburgs Universitair Centrum (LUC) te Diepenbeek, België, waar zij in 1996 het diploma van Kandidaat in de Scheikunde behaalde. Vervolgens heeft zij haar opleiding voortgezet aan de Universitaire Instelling Antwerpen (UIA), waar zij in 1998 het diploma van Licentiaat in de Biochemie behaalde. In het kader van het afstudeertraject, werkte zij van januari 1998 tot mei 1998 aan haar thesis, getiteld 'De rol van apoptose en proliferatie bij het ontstaan van het basaal cel carcinoom', bij de vakgroep Moleculaire Cel Biologie aan de Universiteit Maastricht (UM), onder begeleiding van Dr. G. Schaart en Prof.Dr. F. Ramaekers. In oktober 1998 werd dit onderzoek vervolgd in het kader van een AIO-schap onder begeleiding van promotoren Prof. Dr. M. Neumann en Prof.Dr. F. Ramaekers en co-promotor Dr. G. Krekels. Sinds 6 mei 2003 is zij werkzaam bij Cancer Research UK, Skin Tumour Laboratory in Londen.

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Bax expression and growth behavior of basal cell carcinomas

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British Journal of Dermatology (2003): 148 pp 102-109

The garlic derived organosulfur component ajoene decreases basal cell carcinoma tumor size by inducing apoptosis

C.M.L.J. Tilli, A.J.W. Stavast-Kooy, J.D.D. Vuerstaek, M.R.T.M. Thissen, G.A.M. Krekels, F.C.S. Ramaekers, H.A.M. Neumann
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Under revision (British Journal of Dermatology)

Expression of pro- and anti-apoptotic members of the Bcl-2 family and associated proteins in normal human skin and basal cell carcinoma

C.M.L.J. Tilli, H.A.M. Neumann, F.C.S. Ramaekers
Accepted for publication (Experimental Dermatology)

Latin (botanical) name: *Allium sativum*
Common names: Garlic, Poor Man's Treacle

Garlic Soup (The French Kitchen, Harris & Warde)

4tbsp olive oil
5 whole garlic bulbs
25g flour
1.5l hot water
4 sprigs of thyme
sea salt
freshly ground black pepper
100g thin vermicelli
large bunch parsley

Heat the oven to 140°C

Drizzle the oil over a roasting tray and put cut garlic bulbs on it, roast 1-1.5 hours until soft and golden. Remove garlic from oven and squeeze out the soft buttery flesh. Put it in a saucepan with juices from roasting tray and mash with a spoon. Over a medium heat, mix in the flour so it absorbs the oil and makes a roux. Slowly pour in the hot water, stirring constantly. Add thyme and seasoning and simmer for 20 minutes. Remove from heat, discard thyme sprigs and use a blender to make the soup smooth. Return to the heat, add vermicelli and cook for 3 minutes. Add parsley just before serving.