Growth characteristics of basal cell carcinoma

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Growth characteristics of basal cell carcinoma

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Marcus Emile Johannes Maria Verhaegh

Promotores

Prof. dr. H.A.M. Neumann

Prof. dr. J.W. Arends

Co-promotor

Dr. R. Hoekzema

Beoordelingscommissie

Prof. dr. J.J. Manni (voorzitter)

Dr. A.M. Duijvestijn

Dr. V. Noordhoek Hegt (Sint Franciscus Gasthuis, Rotterdam)

Prof. dr. F.C.S. Ramaekers

Prof. dr. B.J. Vermeer (Universiteit Leiden)

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*"There are more things in Heaven and Earth,
Horatio,
than are dreamt of in your philosophy"*

William Shakespeare

Aan mijn Ouders
Voor Jesje, mijn steun in het schijnbaar onbereikbare

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General introduction

Introduction

Basal cell carcinoma (BCC) is the most common type of malignancy seen today and its incidence continues to increase. BCCs are slow growing tumors that rarely metastasize or cause death but can result in extensive morbidity through local invasion and tissue destruction. The cell of origin is probably an epidermal pluripotent stem cell, and there is a range of histologic subtypes, including nodular, superficial and infiltrating. Most BCCs are managed by simple excision, cryosurgery, curettage and electrodesiccation, and radiation therapy, but some primary and recurrent carcinomas present a more difficult challenge. Mohs' micrographic surgery (MMS) is the most accurate method of removing these skin cancers and achieving the highest cure rates.

History

BCC has left evidence of its existence ever since 4000 years ago. By examining mummified skeletons from the Dynastic period in Asyut, an Egyptian city on the left bank of the Nile, Satinoff and Wells found bony manifestations (including jaw cysts, syndactyly, polydactyly and bifid ribs) related to the nevoid basal cell carcinoma syndrome.¹

Despite the antiquity of the lesions, nosologic description of BCC is far more recent. The term *noli-me-tangere* is said to be identified with BCC.² It derives from the Latin, literally "I do not wish that I touch" or, more prosaically, "touch-me-not". The Latin is found in the Vulgate Bible, John 20:17, "dicit ei Iesus, noli me tangere, nondum enim ascendi ad Patrem meum" (translation: "Jesus said to them, 'Do not touch me,' for I have not yet ascended to the Father").³

In the 14th century, the great French surgeon, Guy de Chauliac (circa 1300 to 1368), attendant to popes and kings, referred to ulcerated chancres of the face as "commonly called *noli me tangere*" and recommended a variety of topical remedies including honey, which has antibacterial properties.⁴

Jacques Daviel (1696 to 1762), the oculist of Louis XV, wrote in 1754 a treatise on the surgical treatment of *noli-me-tangere*, in particular of the eyelids, nose, angle of

the eye, and adjacent tissue. He made radical excisions for the time, often leaving the patient disfigured but functioning.⁵

Daviel's work was followed in France by a young doctor's thesis in 1806; *Recherches on the cancer of the skin of the face, called by the Ancients Noli-me-tangere*. The author, P.J.L. Bouchet, argued that facial skin cancers were the least dangerous of all skin cancers, that they were usually a primary lesion, that when removed they rarely returned, and that if untreated they would slowly continue to grow.⁶ In 1827, Arthur Jacob, member of the royal college of surgeons in Ireland, described an ulcer of peculiar character, which attacks the eye-lids and other parts of the face. He tried ointments containing mercury, lead, zinc, copper, arsenic, sulphur, tar, cicuta, opium, belladonna, nitrate of silver and acids, without arresting for a moment the progress of the disease. He concluded, the sooner it is completely expired by the knife, the better chance is afforded the patient of relief from a most distressing and fatal malady.⁷

Many authors followed Jacob in producing clinical descriptions of BCC. In 1902, E. Krompecher clearly delineated this tumor from other epithelial tumors.⁸ Many synonyms have been used for this tumor, e.g. Jacob's ulcer, Krompecher's tumor, rodent ulcer, adnexal carcinoma, carcinoid of the skin, basalioma, basal cell epithelioma, non-cornifying epithelioma and basal cell carcinoma.

Pathogenesis

BCCs almost never occur on mucosal or palmar/plantar surfaces.⁹⁻¹¹ This observation has led to the suggestion that BCCs develop only in epidermis in which pilosebaceous units are present. Several epidemiologic studies have found that the nose is the most prominent site for BCCs to develop.^{12,13} Histologic studies have noted an origin from both follicular and interfollicular sites.¹⁴⁻¹⁶ Recent immunocytochemical work noted identical keratin profiles in BCCs and the outer root sheath of hair follicles, suggesting some etiologic connection.¹⁷

BCC is a neoplasm composed of usually undifferentiated yet quite pluripotent cells, and this suggests a possible etiologic relationship with stem cells.^{18,19}

The cutaneous carcinogenesis process usually is thought to involve the multisteps of initiation, promotion, and conversion.²⁰ However, in BCC development, there is no clinically evident "promotion" stage. It seems that BCCs appear to arise de novo and not from any precursor lesion.

BCC has become significantly more common in recent years. An important cause of this increasing incidence is presumed to result from increased ultraviolet (UV) radiation exposure.

Increased BCC incidence is seen in Caucasian individuals with fair skin, poor tanning ability, blond hair, blue eyes, and male sex, and incidence increases with increasing age and significant lifetime exposure.²¹⁻²⁴

Over 80% of tumors occur on the head and neck, the areas of greatest sun exposure. However, up to 33% of BCCs on the head and neck occur in areas with minimal sun exposure.^{25,26} Therefore causative factors other than UV-radiation have to be considered, like ionizing radiation, chemical carcinogens and possibly infection with human papillomaviruses.²⁷ Moreover, superficial BCCs differ from other subtypes of BCC by occurring more commonly on the trunk and in younger patients.^{27,28} The differences in the age and body site distribution between superficial BCC and the other subtypes of BCC may indicate that they vary in their causal relationship to sunlight. The development of superficial BCC may be more strongly related to intermittent sunlight exposure, whereas the other subtypes of BCC may be related more strongly to cumulative sunlight exposure.

BCC metastasizes very rarely (0.0028% - 0.55%).²⁹ There usually is a long interval between diagnosis of BCC and metastasis, averaging 9 years. Once metastatic disease is diagnosed, survival is brief, averaging 8 months. No characteristics predictive of metastasis have been noted.²⁹ Why BCC metastasizes so rarely is unknown. The most common theory is that BCC is a "stromal-dependent" tumor; in the absence of its stromal component, growth of the malignant cells does not continue.^{30,31}

Molecular genetics

Neoplastic transformation of human cells is believed to result from a series of genetic alterations that lead to progressive disorder of growth and differentiation. This multistep process involves activation of oncogenes and/or inactivation of tumor suppressor genes.³² There are only a few studies showing oncogene activation in BCCs. The *fos* oncogene shows high expression in some infiltrative BCCs but low expression in well-circumscribed BCCs.^{33,34} Several investigators have shown *H-ras* and *K-ras* point mutations in a small fraction of BCCs.³⁵⁻³⁷

These data are suggestive that oncogene activation may play only a secondary role in BCC pathogenesis.

Bcl-2, an oncogene that protects cells from programmed cell death, is expressed at high levels in BCCs, but rearrangements of the gene have not been demonstrated in this tumor.³⁸

Tumor suppressor genes normally exert a negative control on cell growth. Inactivation of both homologues ("two hits") is required for a growth-promoting effect. *p53* is a tumor suppressor gene that plays a role in carcinogenesis in a wide variety of tissues.³⁹ The observed loss of heterozygosity for alleles on chromosome 17p in

BCCs was presumed to be related to inactivation of *p53*. Direct sequencing of the *p53* gene revealed mutation of at least one allele in approximately 50% of BCCs.⁴⁰⁻⁴² Until now, no relationship between *p53* mutation and tumor size, histology, or recurrence, has been identified.

Constitutional tumor suppressor gene mutations underlie a large number of hereditary cancer predisposition syndromes³², and key evidence that BCCs may arise through inactivation of a tumor suppressor gene came from studies of hereditary predisposition to BCCs. Approximately 0.5% of BCC cases are attributable to the nevoid basal cell carcinoma syndrome (NBCCS),⁴³ an autosomal dominant disorder characterized by multiple BCCs, keratocysts of the jaw, and pits of palms and soles.⁴⁴ On the basis of the clinical behaviour of these BCCs, several investigators have suggested that NBCCS fits a two-hit model;⁴⁵⁻⁴⁷ i.e., tumors develop in cells sustaining two genetic alterations. The first alteration or hit is the inheritance of a mutation in a tumor suppressor gene, and the second hit is inactivation of the normal homologue by environmental mutagenesis or random genetic rearrangement. Sporadic BCCs would arise in cells that underwent two somatic events resulting in inactivation of the NBCCS gene.

Tumor deletion and gene mapping data support the hypothesis that NBCCS is caused by mutation in a tumor suppressor gene on chromosome 9 (allelic loss for chromosome 9q22).⁴⁸

A human sequence (PTCH) with strong homology to the *Drosophila* segment polarity gene, *patched*, was isolated from the NBCCS region and shown to be expressed in many of the tissues affected in NBCCS patients.⁴⁹⁻⁵¹ The *Drosophila* version of *patched* is a cell surface molecule that may be involved in cell-cell communication. In addition, *patched* is known to suppress the expression of various growth factors.

Most likely the NBCCS gene is a "gatekeeper", the loss of which is needed to start the process of carcinogenesis;^{52,53} and *p53* mutations possibly contribute to the malignant characteristics of tumors.

Biologic characteristics

Factors that influence the rates of cell division and apoptosis together determine the growth rate of BCC. BCCs are typically slow growing tumors, often taking months to years before reaching significant proportions.⁵⁴ Studying tumor cell proliferation by means of *in vivo* injection of 3H-thymidine in patients with BCCs, Weinstein and Frost calculated the BCC tumor cell cycle to be 217 hours, or a tumor doubling time of only a few weeks.⁵⁵ Such a relatively rapid growth rate is at odds with the clinically observed, characteristically slow BCC doubling time of months to years. BCC tumors have been estimated to grow at a rate of between

1 mm and 6 mm annually.⁵⁶⁻⁵⁸ More recently, however, Terz and coworkers, using *in vivo* BCC labelling techniques, demonstrated that between 5% and 40% of tumor cells were arrested for variable time periods in the G2 or G0 phases of the cell cycle.⁵⁹ They contended that such a large compartment could account for the observed slow growth rates of these tumors. Heenan and Galand later reported a tumoral prolongation of the S (DNA synthesis) phase of the cell cycle, an effect which could also contribute to the observed slow growth rates of BCCs.⁶⁰ Finally, supporting the notion that a BCC typically grows slowly, because a large percentage of its cells proliferate either slow or not at all, Grimwood et al, using 3H- thymidine in the nude mouse model, found that labelled material was primarily taken up by those cells located at the periphery of a BCC nodule, rather than by those located more centrally.⁶¹ In more aggressive histologic subtypes, such as infiltrative BCCs, mitotic figures are more common and are observed at random throughout the tumor islands.^{62,63}

Because tumor growth is the net result of proliferation versus cellular degeneration, induction of cell degeneration could also be an important feature contributing to the slow growth of BCC. In indolent BCC subtypes, growth rates may be partially determined by apoptosis, a programmed form of cell death that can regulate cell populations, while in aggressive types of BCC, apoptosis may be inhibited.^{64,65}

The protein product of the oncogene *bcl-2* has been shown to inhibit apoptosis.⁶⁶ In a recent study in which *bcl-2* protein expression was used as an indicator of apoptotic inhibition and in which immunocytochemical techniques were used, BCCs stained strongly for the presence of *bcl-2*.⁶⁵

Cellular immunology

The mechanisms of anti tumor-immune response are rather complex and are as yet neither fully elucidated nor understood. However, several subpopulations of T cells, cells of the monocyte-macrophage lineage, natural killer (NK) cells, B cells producing specific and natural cytotoxic antibodies have been implicated in the host's anti-tumor immune response.

The role of the immune system in controlling BCC development and growth is still under debate.

Systemic immunity: The incidence of nonmelanoma skin cancer increases with age. Decreasing systemic immune function and increased exposure to carcinogenic agents, especially UV light, may play a role. Depression of cell-mediated immunity begins at puberty and becomes most pronounced after the age of 50 years.⁶⁷

The immunosuppressed status of patients appears to play only a modest role in increasing BCC incidence. Several studies of renal transplantant populations and chronic PUVA users have noted an increase in cutaneous SCC incidence in these immunosuppressed patients of 20 to 30 times the incidence compared to the normal population.⁶⁸⁻⁷⁰ However, increases in BCC incidence in these studies were much more modest, ranging from 1.4 to 5 times the normal incidence. The clinical behaviour of BCCs in immunosuppressed patients does not appear to be significantly more aggressive than in normal populations.

Immunological studies in patients with BCC have demonstrated several abnormalities. Dellon et al observed an absence of infiltrate and a low T-cell level in the peripheral blood of patients with BCCs of large size (2 cm in diameter) and aggressive growth pattern.^{71,72} Two other groups, however, found normal T cell levels in 80 patients with BCCs, although in a small subset of patients with metatypical BCC tumors, levels were depressed.^{73,74}

Local immunity: Only around one third of BCCs which are excised with histologically proven positive surgical margins are estimated to recur.⁷⁵ In addition, spontaneous partial regression is not an uncommon phenomenon in BCC. Curson et al noticed active regression zones in 14% of investigated BCCs.⁷⁶ Immune-mediated effects may be involved in both of these phenomena. Hunt et al, suggested that activated CD4+ cytokine-secreting cells are important in the regression of BCCs.⁷⁷

BCCs are frequently (about 60%) associated with a peritumoral mononuclear infiltrate. Until now, the function of this inflammatory infiltrate and its possible role in the control of tumor growth is unclear. Typically, inflammatory cells are most dense at the lateral and inferior borders of the tumor, without touching or infiltrating the BCC tumor lobules.⁷⁸ The infiltrate consists primarily of T lymphocytes with a predominance of the CD4+/T-helper over the CD8+/T-cytotoxic subset, similar to that found in typical delayed hypersensitivity reactions.⁷⁸⁻⁸⁰ Natural killer cells, considered to be the first nonspecific line of defence against tumors, and B-cells are seen in much lower numbers.

Besides identifying the immunoregulatory subsets of lymphocytes, the functional activation status also is of considerable importance. Different stages of lymphocyte activation are characterized by the appearance of specific cell surface antigens. Interleukin 2 receptor (IL2R) is expressed during the early stages of activation.⁸¹ Transferrin receptor expression by lymphocytes occurs later than IL2R, approximately 48 hours after activation.⁸² The class II major histocompatibility complex molecule human lymphocyte antigen (HLA)-DR appears late at between 5 and 7 days.⁸² The expression of the antigen recognized by the antibody OKT10 occurs intermediate in time between that of the transferrin receptor and HLA-DR.^{82,83} Markey et al demonstrated expression of activation antigens in six BCCs. The class II molecule HLA-DR was expressed by 58% of the infiltrate, with fewer lym-

phocytes variably staining for IL2R (13%), transferrin receptor (16%), and OKT10 (22%).^{84,85}

The presence of an activated infiltrate around BCCs has been suggested to explain in part the slow growth of this tumor. However, similar findings of HLA-DR, transferrin receptor, and IL2R expression are demonstrated also in benign and premalignant tumors. Furthermore, the lack of qualitative or quantitative differences in the infiltrates accompanying divergent tumors suggests that the activated infiltrate alone does not seriously affect the biologic behavior of the tumor.

The fact that also a considerable number of OKT10+ T cells were observed in the peritumoral infiltrate around BCCs, possibly is of more importance. Especially because this subset of activated T cells is typically found in the presence of malignant skin tumors but not in inflammatory dermatoses.^{84,86}

Finally, a selective expression of specific $\alpha\beta$ T-cell receptors by BCC peritumoral T cells is demonstrated.⁸⁷ T cells with $\alpha\beta$ T-cell receptors recognize antigens and are responsible for a specific immune response.

In order to control the proliferation of the tumor by the surrounding cellular infiltrate, expression of adhesion molecules on tumor cells is required. The expression of class I major histocompatibility complex (MHC) antigens by BCC tumor cells is decreased compared with normal skin, and class II antigens are not expressed by BCC tumor cells at all.⁸⁸⁻⁹¹ Regarding adhesion molecule expression in immune-tumor cell interactions, lymphocyte function associated antigens LFA-1 and LFA-2 are expressed on T lymphocytes, and these interact with their complementary ligands located on tumor cell surfaces, intercellular adhesion molecule-1 (ICAM-1) and the widely distributed surface molecule LFA-3, respectively.⁹² Several studies demonstrated that ICAM-1 and LFA-3 are not expressed by BCC tumor cells at all.^{90,93}

The lack of constitutive ICAM-1 expression supports the contention of others that BCCs are less immunogenic than other cutaneous tumors because adhesion molecules are essential to cytotoxic killing functions.^{73,74}

The inability of BCC tumor cells to express adhesion molecules is not absolute. Taylor et al presented a study where they demonstrated induction of ICAM-1 expression on tumor cells after BCC tissue had been incubated *in vitro* with IFN- γ .⁹⁴ Interestingly, tumor cells exposed to IFN- α remained negative for ICAM-1 and LFA-3.

Clinical presentation

A typical BCC has distinctive morphologic features that make it readily recognizable. A pearly, telangiectatic papule occurring on the cheek of an elderly Caucasian man presents no significant diagnostic dilemma. Yet, the presentations of

BCC are as varied as the tumor is common. For example, a superficial BCC is characterized by a red, slightly scaling, well-demarcated eczematous-appearing patch with a thread-like pearly rolled border. The morpheaform BCC is an atrophic, white, sometimes slightly eroded or crusted plaque that often looks like a scar.

BCC is the most common malignant tumor in humans, and skin cancers currently constitute approximately one third of all diagnosed cancers. The incidence of BCC is increasing worldwide, likely under the influence of sunbathing behaviour, aging and a decrease in stratospheric ozone.

The reported incidence of BCC in Caucasians in the United States ranges from 185 to 422 cases per 100.000 population.⁹⁵ According to the Skin Cancer Foundation, one in six Americans will develop non-melanoma skin cancer (NMSC) during his or her lifetime. Although less data exist for African-Americans, one reported figure is 3.4 per 100.000.⁹⁶ In Australia, incidence figures between 672-760 per 100.000 are reported.^{97,98} In the Netherlands (15 million inhabitants), approximately 18.000 BCCs (incidence of 120 per 100.000) were diagnosed in 1994, with an expected increase of 2.700 BCCs yearly.⁹⁹

Important BCC demographic variables include age, gender, degree of pigmentation, history of previous BCC, associated skin lesions, history of previous trauma, and general medical history.

BCC becomes increasingly common with age, with an average age somewhat older than 60.¹⁰⁰ However, BCC has been documented in several collected series in patients younger than age 20.¹⁰¹⁻¹⁰³

Most studies show BCC to be more common in men. However, some recent work in younger populations suggests that this trend may be reversing, probably related to changing habits among young women.^{104,105}

Intrinsic pigmentation is clearly one of the best predictive risk factors for BCC. It is estimated that BCC accounts for no more than 8% of all skin cancers in African-Americans, this in contrast with Caucasians in which approximately 70% of all skin cancers are BCCs.^{106,107}

It is not uncommon for patients to present with more than one BCC tumor. One study reported that the incidence of multiple, coexisting tumors was 13%.¹⁰⁸ Robinson performed a 5-year prospective study of 1000 BCC patients; 36% developed a second BCC and 15% developed a third tumor in the same time period.¹⁰⁹ Other studies calculated for individuals with at least one non-melanoma skin cancer an estimated risk of 17% within 1 year, 35% within 3 years, and 50% within 5 years to develop an additional nonmelanoma skin cancer.¹¹⁰⁻¹¹²

BCCs have been reported to develop in traumatic settings, the scars of lupus vulgaris, chronic stasis ulcers, lesions of epidermolysis bullosa, epidermal inclusion cysts, hemangiomas, pilomatricomas, porokeratoses, and nevi flammeus.¹¹³⁻¹¹⁸ Some of these associations may be random. In some cases, however, the associa-

tion between a skin lesion and a BCC is not at random. The risk of BCC development is clearly increased in persons with dermatofibroma, nevus sebaceus and linear unilateral basal cell nevus. BCC-like changes occur in 2% to 8% of dermatofibromas.¹¹⁹ The neoplastic potential of these basaloid proliferations overlying dermatofibromas was once doubted. However, recent data using antikeratin antibodies, $\beta 2$ microglobulin and *bcl-2* studies, have demonstrated that the BCC-like basaloid changes are immunohistochemically identical to sporadic BCCs.¹²⁰⁻¹²² BCCs have been reported to originate in 5% to 20% of sebaceous nevi.^{123,124} Both dermatofibromas and nevus sebaceus are characterized by onset of BCCs in young patients. Linear unilateral basal cell nevus is a very rare, non-genetic disorder first described by Carney in 1952.¹²⁵ Clinically, it presents as an extensive, unilateral, linear or zosteriform eruption of BCCs, usually at birth.¹²⁶ BCCs are also clearly associated with several genodermatoses, including the nevoid basal cell carcinoma syndrome, Bazex's syndrome, Rombo syndrome, albinism, and xeroderma pigmentosum.¹²⁷⁻¹²⁹ Finally, BCC has a well known association with previous ionizing radiation and inorganic arsenic.^{130,131}

Histology

BCC is recognized histologically by the presence of multiple islands, nests, or cords of basaloid tumor cells with peripheral palisading of nuclei and variable tumor-stromal separation artifact. The tumor generally arises from the overlying epidermis or adjacent follicular epithelium. The basaloid tumor cells have uniform, round to oval, slightly hyperchromatic nuclei, coarsely clumped chromatin and scant cytoplasm. Nucleoli are inconspicuous and mitoses are variable. The surrounding stroma is characteristically mucinous, with a variable fibroblastic and inflammatory component.^{132,133}

A wide variety of BCC histopathologic subtypes have been described.¹³⁴ Many are purely descriptive and have not been shown to affect growth rate. This is particularly true of those tumors differentiating toward appendageal structures.

Lang and Maize were the first to clearly define five BCC subtypes with important clinical implications;¹³⁵ superficial, nodular, micronodular, infiltrating, and morpheaform. They also described the frequent overlap of histologic patterns within a given tumor, i.e., the mixed-type (Fig.1).

Superficial BCC has also been termed superficial multifocal, superficial multicentric, or superficial spreading BCC. This type accounts for approximately 10% of all BCCs.¹³⁶ Histologically, it is characterized by multiple small buds of basaloid tumor cells that arise from the epidermis and extend into the superficial dermis. All nests are in continuity with the epidermis, although occasionally there is arti-

Figure 1. Growth patterns of BCCs.

factual separation resulting from the plane of a cut. When tumor nests have no connection with the epidermis, the BCC should not be classified as superficial BCC. Superficial BCC is unique in that its lateral margins are notoriously difficult to delineate histologically. In three-dimensional reconstructions, the tumor buds have been shown to form anastomosing or interconnecting complexes.¹³⁷ In two-dimensional histologic sections, however, they often appear as multiple, small, independent tumors, separated by skipped areas of involvement.¹³⁸ Therefore, the pathologist may erroneously inform the clinician that the lesion has been removed completely because the lateral borders appear to be tumor-free, when, in fact, the lateral borders represent the skipped areas.

Nodular BCC, also known as solid, macronodular, or well circumscribed BCC, is the most frequent architectural pattern encountered, accounting for 50% to 70% of cases.^{133,136} Nodular BCCs show features that have been considered classic for

BCC, including well circumscribed, large rounded islands of tumor cells with a smooth, palisaded peripheral border and stromal retraction. Larger or long-standing lesions that ulcerate are termed nodulo-ulcerative. There may be an “adenoidal” pattern of the large tumor islands or central tumor necrosis leading to the cystic changes described in some tumors.^{132,133}

Micronodular BCC is similar to nodular BCC except that the tumor nests are predominantly small and rounded. These smaller tumor islands often approximate the size of hair bulbs.^{139,140} The peripheral palisading may not be as well developed as in nodular BCC and the stroma is often more collagenous, rather than myxoid. The micronodular variant is characterized by significant subclinical extensions, and an infiltrate-pattern widely through the dermis or subcutis.

Morpheaform or sclerosing BCC shows thick, scarlike collagen (sclerosis) in the surrounding dermis, with the tumor cells appearing to “force their way” through this dense connective tissue.

Infiltrating BCCs have tumor islands of small but variable size. The border of the islands is not smooth; it has irregular, spiking, or jagged projections. Some islands extend out into a single-cell pattern, much like a morpheaform BCC but without the sclerosis. Stromal retraction is often not present. Most infiltrative BCCs are characterized by a variable nodular component in the superficial part of the lesion, but the infiltrative portion constitutes the majority of the tumor. Together, the morpheaform and infiltrating BCCs, account for approximately 10% to 15% of all BCCs.

It appears that morpheaform and infiltrating patterns are most aggressive (i.e. an infiltrative rather than an expansile pattern of growth), the nodular and superficial are the least aggressive, and the micronodular pattern occupies a place somewhere in between.

Many tumors have a mixed tumor type, i.e., two or more histologic subtypes represented in the same tumor. In one study, such a mixed pattern was seen in 38.5% of the cases.¹³⁹ In these tumors, clinical behaviour reflects the most aggressive subtype found within the tumor. Often the most aggressive pattern is present at the base of a tumor and may be missed if a superficial biopsy is taken.¹³⁹ There are many other histologic subtypes of BCC. They often further define a particular lesion, by describing the pattern or direction of differentiation, e.g., toward an adnexal structure, or by noting specific cytologic features. To date, there are no data suggesting that these variants affect prognosis in any way, with the possible exception of basosquamous carcinoma. The existence of this last lesion as a subtype of BCC or as a transitional form between BCC and squamous cell carcinoma (SCC) is controversial and has been hotly debated.^{141,142} The criteria used to define basosquamous carcinoma vary widely in the literature. Those lesions that have a distinct admixture of conventional BCC and classic SCC may be classified as basosquamous carcinoma. The term “metatypical BCC” has also been applied to tu-

mors with a mixed basaloid and squamous histology and, thus, is often used interchangeably with basosquamous carcinoma. The reported incidence of basosquamous BCC type is 2% to 3%,^{141,143} but differentiating basosquamous carcinoma from keratotic BCC, BCC with squamous metaplasia, and “collision” tumors may be quite problematic, resulting in an overdiagnosis of basosquamous carcinoma. Nevertheless, the concept of a cutaneous neoplasm that represents a continuum between BCC and SCC is an important one, since the literature and also our own experience suggests that basosquamous carcinoma may behave in a more aggressive fashion than conventional BCC, with a higher rate of local recurrence and metastasis.^{132,141,143,144}

Some other histologic subtypes are: adenoid BCC (an adenoid pattern is seen in 1% to 7% of BCCs, especially in association with nodular BCC),¹³⁶ pigmented BCC (pigmentation is present in 6% to 7% of BCCs),^{136,145} fibroepithelioma of Pinkus (characterized by multiple, long, thin, anastomosing strands of basaloid cells),¹⁴⁶ BCC with follicular differentiation (keratotic BCC and infundibulocystic BCC),¹⁴⁷ BCC with sebaceous differentiation (sebaceous epithelioma), BCC with apocrine differentiation, BCC with eccrine differentiation (this type of tumor must be distinguished from eccrine carcinoma, adenoid cystic carcinoma, and microcystic adnexal carcinoma), adamantinoid BCC, granular cell BCC, pleomorphic BCC, clear cell BCC, signet-ring BCC and BCC with neuroendocrine differentiation.

Finally, there are many cutaneous neoplasms that may histologically simulate BCC. Some selected histologic simulants are: trichoepithelioma, desmoplastic trichoepithelioma, basaloid follicular hamartoma, folliculocentric basaloid proliferation, microcystic adnexal carcinoma, peripheral ameloblastoma, and cloacogenic carcinoma.

Features associated with recurrence

Although most BCCs are cured by initial treatment, incomplete removal will result in tumor persistence, which may further result in continued tumor growth (approximately 33%), often with substantial morbidity, and occasionally, mortality.¹⁴⁸⁻¹⁵⁰ Most BCCs that recur do so within 3 years of treatment.¹⁵¹ However, some BCCs recur many years later. Rowe et al combined recurrence statistics from several studies and concluded that 30% of recurrent BCCs recurred in the first year after treatment, 20% in the second year, 16% in the third year, 8% in the fourth year, 8% in the fifth year, and 18% of BCC recurrences occurred 6 to 10 years after treatment.¹⁵²

Perhaps tumor dormancy and/or decreases of peripheral T-cell levels may play a role in the timing of BCC tumor cell proliferation.¹⁵³ The type of surgical closure

may also influence the time it takes for a BCC recurrence to become manifest. In a study by Richmond et al, most recurrent BCCs appeared by the end of the first two years after surgery if the wound was closed by a split thickness skin graft or a full thickness skin graft, and by the end of the fourth year if closed by a skin flap.¹⁵⁴

The important variables in a primary BCC influencing whether the tumor will recur after treatment include anatomic location, histologic subtype, size, and previous treatment.¹⁵⁵⁻¹⁵⁷

The anatomic sites known to be more difficult to eradicate the tumor completely include; the scalp, ears, eyes, nose including the paranasal areas, mouth, nasal vestibule, auditory meatus, and rectum.¹⁵⁷⁻¹⁵⁹ In addition, the so-called “embryonic fusion” planes, located in such areas as the preauricular and postauricular sulci, the paranasal regions, and the inner canthi, allow deep extensions of BCCs because of the presence of noncontinuous and nonuniform subcutaneous tissues.^{156,160,161}

Infiltrative/morpheaform BCC is often the subtype of BCC found in situations of recurrence,^{160,162,163} it is also the histologic subtype most prone to recur after treatment,^{164,165} and the one most prone to be associated with disease-positive surgical margins and incomplete excision.¹⁶⁶

Large BCCs (> 2 cm) are more difficult to cure than small BCCs.^{167,168} Large BCCs have, by definition, been allowed to progress and have therefore had the opportunity to grow deeply and peripherally.^{169,170}

Multiply recurrent BCCs have high recurrence rates regardless of the anatomic site or treatment modality used.^{159,171}

Principles of management of BCC

There are a number of options available to the physician for the management of BCC. The most commonly used treatment modalities are surgical excision including Mohs' micrographic surgery (MMS), cryosurgery, radiation therapy, curettage and electrodesiccation. Before undertaking one of these treatments, however, it is important to assess patient and tumor individually, so that the most appropriate therapeutic option may be selected. The patient's age, sex, health, skin color, and degree of sun damage; the tumor's location, size, histologic subtype, and history of previous treatment must all be considered in determining the optimal therapeutic modality. In addition, potential complications, cosmesis, costs, and most importantly, recurrence rates for each approach must be evaluated with respect to each individual patient.

The role of each mode of therapy is summarized in Table I, based on principal indications, advantages, disadvantages, and recurrence rate.¹⁷²⁻¹⁹⁶

Only MMS will be discussed in further detail because this procedure is less widely used in the Netherlands and consequently less known.

It all started when Frederic E. Mohs was a Brittingham Research Assistant to Professor Michael F. Guyer, geneticist and chairman of the Department of Zoology at the University of Wisconsin in the early 1930s. While conducting experiments with various injectable irritants to study the inflammatory response in transplantable rat cancers in normal tissue, they noticed that 20% zinc chloride solution caused tissue necrosis. They also happened to notice that microscopic examination showed well preserved structure of the cells and tissue, the same as if the tissue had been excised and immersed in a fixative solution. Dr. Mohs stated "this observation of *in situ* fixation might have been unrecorded and forgotten if it had not suggested the idea that it might be the basis for a method by which cancers could be excised under complete microscopic control." Skin cancers seemed most amenable to this approach. While he pondered this concept, he realized that vertically cut sections of excised tissue would not be practical because of the large number of sections needed. He also realized that step sections would leave unexamined gaps inbetween. Finally, he conceived of the idea of excising the tissues layer by layer and microscopically examining the entire undersurface of each layer.

Then, Dr. Mohs designed a paste vehicle for zinc chloride which made accurate control of depth fixation possible. The advantages of zinc chloride included good *in-situ* fixation, good penetration, lack of damage to the healing qualities of the underlying tissues, lack of systemic toxicity, safety in handling, and lack of any tendency to increased metastasis.

To Dr. Mohs it seemed logical that an inert granular material would be important as a base to permit the zinc chloride solution to percolate down between the particles like water permeates through sand. However a binder would be necessary to prevent the solution from settling to the bottom of the container. He visited an old-fashioned pharmacy where the 85 year old proprietor introduced him to a black granular antimony or stibnite, and a number of plant extracts including blood root powder *Sanguinaria Canadensis*. The stibnite was a good granular matrix and the *Sanguinaria Canadensis* was an adequate binder. The pharmacist's paste was too soft so after many trials, Dr. Mohs settled upon a 4:1 portion of stibnite and *Sanguinaria Canadensis*. The solution ended up as stibnite 40 grams, *Sanguinaria Canadensis* 10 grams and zinc chloride saturated solution 34.5 ml. This solution was original enough to be patented and Dr. Mohs signed over all rights to the patent to the Wisconsin Alumni Research Foundation for \$1. The completion of the development of the paste was in 1936 and at this time the term "chemosurgery" was coined by Dr. Mohs. In 1941 Dr. Mohs published his first three papers on this new technique.

Table 1. Modalities used for treatment of BCC

Modality	Indications	Advantages	Disadvantages	Recurrence Rate
Surgical excision	<ol style="list-style-type: none"> 1. Primary BCC, Recurrent BCC 2. Well-defined nodular, superficial or infiltrative BCC 3. Size < 2 cm 4. Located in low-risk areas 	<ol style="list-style-type: none"> 1. Histologic verification of the margins 2. Rapid healing 3. Cosmetically acceptable linear scar 4. Scar improving over time 	<ol style="list-style-type: none"> 1. Normal tissue will be sacrificed 2. Proficiency required 3. Risk of bleeding 	<ol style="list-style-type: none"> 1. 0.7-10.1% for primary tumors 2. 11.6-17.4% for recurrent tumors
Cryosurgery	<ol style="list-style-type: none"> 1. Primary BCC 2. Well-defined nodular or superficial BCC 3. Tumor size > 2 cm 4. Trunk, eyelid, ears, nose, medial canthus 	<ol style="list-style-type: none"> 1. Simple to perform 2. Cost-effective 3. Rapidly performed 4. Multiple BCCs 5. Cartilage and lacrimal duct sparing 	<ol style="list-style-type: none"> 1. No histologic verification 2. Slow healing 3. Cure rates are technique-dep 4. May lead to hypopigmentation 	<ol style="list-style-type: none"> 1. 7.5% for primary tumors
Radiation therapy	<ol style="list-style-type: none"> 1. Primary BCC 2. Well-defined BCC 3. Tumor size > 2 cm 4. Lip, eyelid, ear, nose 5. Adjuvant therapy 	<ol style="list-style-type: none"> 1. Cosmetic results 2. Tissue sparing 	<ol style="list-style-type: none"> 1. No histologic verification 2. Time consuming 3. Expensive 4. Slow healing 5. Avascularity of skin 6. More aggressive recurrent tumors 	<ol style="list-style-type: none"> 1. 7.4%-8.7% for primary tumors 2. 9.5-9.8% for recurrent tumors
Curettage and electrodesiccation	<ol style="list-style-type: none"> 1. Primary BCC 2. Well-defined nodular or superficial BCC 3. Size < 2 cm 4. Located in low-risk areas 	<ol style="list-style-type: none"> 1. Simple and rapidly performed 2. Cost effective 3. Multiple BCCs treated in one visit 	<ol style="list-style-type: none"> 1. No histologic verification 2. Low cure rates for high-risk tumors 3. Cure rates are technique dependent 	<ol style="list-style-type: none"> 1. 3.3-7.7% for primary tumors 2. 40% for recurrent tumors
Mohs' micrographic surgery	<ol style="list-style-type: none"> 1. Recurrent BCC 2. Ill-defined BCC 3. Tumor size > 2 cm 4. Located in high risk areas 5. Infiltrative BCC 6. Perineural growth 	<ol style="list-style-type: none"> 1. Examination of 100% of the margins 2. Tissue sparing 3. Final wound closure 	<ol style="list-style-type: none"> 1. Time consuming 2. Expensive 3. Cure rates are technique-dep (high-quality frozen sections) 	<ol style="list-style-type: none"> 1. 1.0% for primary tumors 2. 5.6% for recurrent tumors

The fresh-tissue technique was first published in Dr. Mohs' chapter in Epstein's first edition of *Skin Surgery* in 1956 and in ten articles during the following ten years. Dr. Mohs modified the traditional fixed-tissue technique by abandoning the use of zinc chloride paste, and excised fresh tissue under local anesthesia with immediate checking of the horizontal frozen sections. At the 1969 meeting of the American College of Chemosurgery, Dr. Mohs reported the use of the fresh-tissue technique for 66 BCCs and for SCCs of the eyelid with cure rates of 100%. However it was not until December of 1970 when Dr. Theodore Tromovitch presented a series of 75 patients treated with the fresh-tissue technique, that the technique began to become popular. The fixed-tissue technique has been largely replaced by the fresh-tissue technique over the last 25 years because of several distinct advantages; 1) less pain, 2) greater efficiency, as there is no need to wait for the zinc chloride paste to fix the tissue, 3) less time consuming, as multiple stages can be performed during the same day, 4) increased healing time, as the frozen-tissue technique allows immediate repair of the wound after tumor-free margins are obtained.

In 1967 the American College of Chemosurgery was formed, consisting mostly of Dermatologists. From the 1950s to the 1970s training was informal, lasting from several days to several months. In the 1980s fellowships were formalized to one or two years. In 1986 the American College of Chemosurgery officially changed its name to the American College of Mohs' Micrographic Surgery and Cutaneous Oncology. In June 1990, under the direction of Matthew H. Goodman, then full-time Director of Dermatologic Surgery at the University of California, the American Society for Mohs' Surgery was formed. On April 6th 1990 the European Society of Micrographic Surgery was founded in Estoril, Portugal.

MMS offers the highest cure rates, while maximizing the preservation of normal tissue to simplify reconstruction of the defect. The key difference between MMS and conventional excision lies partly in the surgical technique but mainly in the orientation and histologic visualization of the margins of the specimen. If the margins of specimens are assessed in routinely processed frozen or paraffin sections, the edges generally are examined by means of vertical sections through representative areas of the specimen; this method provides pathologists with sample views of less than 0.1% of the margins of the tissue. The surgeon performing MMS examines 100% of the true surgical margin by using specially embedded and tangentially cut frozen sections.

Aims of the present study

BCC is the most common type of cancer in the Caucasian population. Although mortality from BCC is low, its importance as a public health problem has been

understated. Interest in the etiology, biology, and treatment of BCC has increased recently because of a well-documented worldwide increase in incidence. BCC is defined as a slow growing malignant neoplasm of the skin, often characterized by extensive subclinical tumor extensions and local destruction, leading to mutilation and recurrence after surgical excision. The occurrence of metastasis is extremely rare.

Although much is known about the progression of BCC, explaining this behaviour on a basic biologic level has been difficult.

The main aims of the investigations reported in this thesis were to obtain some explanations for both the histological and clinical growth characteristics of BCC.

Three lines of investigations were followed.

First, we attempted to elucidate the function of the cellular inflammatory infiltrate surrounding BCCs and its possible role in controlling tumor growth. Therefore, investigations were undertaken to obtain insight into the extent of expression of adhesion molecules on both peritumoral endothelial cells and BCC tumor cells. Expression of these adhesion molecules on endothelial cells is essential for recruitment of T cells into the tissue, and expression on tumor cells is required for lymphocyte-target cell interaction (chapter 2).

We also investigated the possible role of cytotoxic T cells and natural killer cells as active participants in immune surveillance towards BCC, by analysis of granzyme B expression. The expression of granzyme B by cytotoxic T cells and natural killer cells correlates with activation and subsequent cytolytic potential of these cells *in vitro* (chapter 3).

The second line of investigation concerned the critical balance between cell proliferation and cell death. Previously, prominent cell death has been suggested as an explanation for the clinically slow growth rate of BCC. Recently, a new class of proto-oncogenes has been defined, which contribute to malignancy by inhibiting programmed cell death. We investigated the expression of the *bcl-2* (B-cell leukaemia/lymphoma-2) proto-oncogene in BCC. *Bcl-2* expression by BCC tumor cells would suggest a neoplastic transformation caused by extended cell survival, and would argue against prominent cell death as a possible explanation for the typically slow growth rate of BCCs (chapter 4).

Moreover, a search for useful markers was undertaken to discriminate BCC from other lesions that are regarded as clinical and histologic simulants. Besides *bcl-2*, staining differences for transforming growth factor-beta and *CD34* were studied, comparing BCC and trichoepithelioma (chapter 5).

Finally, the purpose of the third line of investigations was to obtain clinical information about possible risk factors and subclinical tumor extensions, in order to determine parameters enabling us to: a) distinguish aggressive BCCs, characterized by extensive local invasion and disfigurement, from the more benign type of BCC (chapter 6), and b) propose surgical margins for primary as well as recurrent

BCCs (chapter 7). These guidelines were formulated on the basis of two retrospective studies of 72 and 309 BCCs respectively treated by Mohs' micrographic surgery in our department.

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Chapter 2

Adhesion molecule expression in basal cell carcinoma

M.E.J.M. Verhaegh, R.C. Beljaards, J.C.J.M. Veraart,
R. Hoekzema, H.A.M. Neumann

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Summary

Basal cell carcinomas (BCCs) are frequently associated with a peritumoral mononuclear infiltrate. Until now, the function of this inflammatory infiltrate and its possible role in the control of tumor growth is unclear. Mechanisms controlling endothelial and target cell adhesiveness for leukocytes are important features in the development of a specific local immune response. The expression and distribution of the adhesion molecules ICAM-1, VCAM-1 and E-selectin by microvascular endothelial cells and tumor cells, together with their leukocyte receptors LFA-1, VLA-4 and CLA respectively, were studied in 33 BCCs of different histological subtypes. In normal skin ICAM-1 is expressed by resting endothelial cells, whereas VCAM-1 and E-selectin expression correlates with endothelial activation. The epidermis in normal conditions displays no ICAM-1, VCAM-1, or E-selectin expression. In BCC, endothelial ICAM-1 expression was only slightly increased compared to normal skin, whereas expression of endothelial VCAM-1 and E-selectin was low or absent in all BCCs examined. Peritumoral infiltrates contained mostly LFA-1 expressing lymphocytes, with minimal VLA-4 and CLA positivity. In none of the cases studied adhesion molecule expression of BCC tumor cells was identified.

The lack of significant expression of adhesion molecules on peritumoral vascular endothelial cells and BCC tumor cells is not in favour of specific cell-mediated immunity as an important mechanism in limiting BCC tumor spread.

Introduction

BCC of the skin, the most common human cancer, is a locally invasive tumor that grows slowly and rarely metastasizes.

BCCs are frequently associated with a peritumoral mononuclear cell infiltrate that may represent a specific antitumor response.^{1,2} The infiltrate surrounding BCC tumor islands largely consists of T cells with a predominance of the CD4+/T-helper over the CD8+/T-cytotoxic subset, similar to that found in typical delayed hypersensitivity reactions.^{3,4} Natural killer cells and B-cells are seen in much lower numbers. To enable infiltrating leukocytes to influence tumor growth, these cells need to be recruited from the circulation first. The entry of lymphocytes from the circulation into an organ is mediated by interactions between adhesion molecules on vascular endothelial cells (so called vascular adressesins) and circulating leukocytes (termed homing receptors).⁵ Recent interest has focused upon three cytokine-inducible leukocyte adhesion molecules, designated intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin.⁶⁻⁸ Another area of interest involving adhesion molecules concerns leukocyte-tumor cell interaction. In order to control the proliferation of the tumor by the surrounding cellular infiltrate, expression of adhesion molecules on tumor cells is thought to be required, and it has been suggested that failure to express adhesion molecules would enable malignant cells to escape immunosurveillance.⁹ In the present study, the distribution of a selected panel of adhesion molecules and their T-cell ligands is tested on a group of thirty-three BCCs of either the nodular- and morpheaform type.

Material and methods

Tissue Specimens

Fresh-frozen skin samples were obtained from patients undergoing Mohs' micro-graphic surgery (MMS) for BCC, present on sunexposed areas of the head. The BCCs consisted of 23 with nodular type and 10 with morpheaform type growth, according to histopathological analysis. Samples were immediately frozen in liquid nitrogen and stored at minus 70°C until required. Normal skin specimens (n=5) from various body sites were used as a control.

Immunohistochemistry

Frozen sections, 5 µm, were cut, air dried, and fixed for ten minutes in acetone. Primary antibodies were applied at the appropriate dilution for 30 minutes at room temperature. Dependent on the species of the primary antibodies, the secondary antibody was either a biotinylated goat anti-mouse immunoglobulin or a biotiny-

lated goat anti-rat immunoglobulin (Dakopatts, Copenhagen). In the third step, streptavidin-peroxydase was applied. HRP-enzyme activity was visualized using 3-amino-ethylcarbizole as a chromogen. Sections were counterstained with hematoxylin. For positive controls the following specimens were used: positive patch test reaction biopsy sites (ICAM-1, E-selectin) and in-vitro TNF- α stimulated skin (E-selectin, VCAM-1).

Negative controls included omission of the primary antibody and substitution of the primary antibody by an irrelevant one. Primary antibodies used are summarized in Table I.

Table I. Monoclonal antibodies used in the study.

Clone	Specificity	Dilution	Source
MEM111	ICAM-1	1:1000	SB
CLB-LFA-1/2	LFA-1	1:150	CLB
ENA-1	E-selectin	1:200	SB
HECA 452	CLA	1:25	PDFU
4B9	VCAM-1	1:2250	Harlan
HP2/1	VLA-4	1:100	IT

(SB, Sanbio, Uden, The Netherlands; PDFU, Pathology department Free University, Amsterdam, The Netherlands; CLB, central laboratory of the Red Cross blood transfusion service, Amsterdam, The Netherlands; IT, Immunotech, Marseille, France; 4B9 was kindly provided by Dr.J.Harlan, Seattle, USA).

Quantification

Expression of endothelial ICAM-1, VCAM-1, and E-selectin was quantified according to the following criteria: 0, no staining; +/-, weak focal staining; +, moderate staining of whole vessels; ++, strong staining of whole vessels. The extent of MoAb staining of leukocytic infiltrates was quantified as follows: 0, no staining; +/-, 0-25% positively stained cells; +, 25-50% positive cells; ++, 50-75% positive cells, and +++, > 75% positive cells.

Results

In 15 cases (solid-type n=6, morpheaform type n=9) of the 33 BCCs studied, only a discrete inflammatory infiltrate was present around the tumor islands. The remaining cases (solid-type n=17, morpheaform n=1) displayed a more prominent lymphocytic infiltrate especially perivascularly and at the base of the tumor. In none of the cases lymphocytes were found infiltrating the BCC tumor nests.

In normal skin moderate ICAM-1 expression (+) of whole vessels was found, whereas endothelial E-selectin and VCAM-1 stained negative (0).

When compared to normal skin, increased endothelial ICAM-1 expression (+/++) in BCC was mainly restricted to those peritumoral areas, where a prominent lymphocytic infiltrate was present (Fig. 1a).

In BCC tumor specimens, endothelial VCAM-1 and E-selectin expression was either undetectable or weakly positive (+/-) with granular staining of individual endothelial cells (Fig. 1b,c). No endothelial staining differences between solid and morpheiform BCC were observed. The peritumoral infiltrate contained mostly LFA-1-expressing cells (++), with fewer lymphocytes variably staining for VLA-4 (+/-) and CLA (+/-). The results are summarized in Table II.

In none of the cases examined ICAM-1, VCAM-1 or E-selectin expression could be identified on tumor cells or overlying benign epidermal keratinocytes.

Figure 1a. BCC stained for ICAM-1. Tumor cell expression of ICAM-1 is absent whereas endothelial cells show enhanced ICAM-1 expression. (x 200). **b.** BCC stained for VCAM-1. Moderate staining for VCAM-1 is observed on vascular endothelium. (x 200). **c.** BCC stained for E-selectin. Only minimal staining for E-selectin is observed on vascular endothelium. (x 200).

Table II. Distribution and staining intensity of vascular adhesion molecules (ICAM-1, VCAM-1, E-selectin) and their T-cell ligands (LFA-1, VLA-4, CLA) in thirty-three basal cell carcinomas.

Antibody	Lymphocytes	Endothelium
ICAM-1	...	+/++
VCAM-1	...	+/-
E-selectin	...	+/-
LFA-1	++	...
VLA-4	+/-	...
CLA	+/-	...

Discussion

In the last decade, several reports concerning the local immune response in BCC have attempted to elucidate the function of the cellular inflammatory infiltrate surrounding these skin cancers and their possible role in controlling tumor growth.¹⁻³ A tumor-specific cellular immune response is considered to be associated with adhesion molecule expression on endothelial cells allowing recruitment of T cells into the tissue, and on tumor cells for lymphocyte-target cell interaction.⁹

At least five molecular pathways operate to recruit T cells to the peripheral tissue through endothelial cell/T-cell interactions that include ICAM-1 and ICAM-2/LFA-1, VCAM-1/VLA-4, E-selectin/CLA and hyaluronic acid/CD44 pathways.¹⁰

ICAM-1, which is inducible by IFN- γ and TNF- α , is expressed by a wide variety of cells, including resting endothelial cells and binds virtually all circulating white cells.^{6,7} E-selectin and VCAM-1 expression correlates with endothelial cell activation and these molecules are both inducible by TNF- α and IL-1.¹¹ VCAM-1 contributes to selective recruitment of T cells and eosinophils but not neutrophils,¹² whereas E-selectin functions as a tissue-selective endothelial cell-adhesion molecule at sites of chronic inflammation and is thought to be important for recruitment of skin-homing T-lymphocytes expressing the cutaneous lymphocyte-associated antigen (CLA).^{13,14}

First, recent literature concerning adhesion molecules in BCC will be summarized. Taylor et al, who studied thirty BCCs, demonstrated negative ICAM-1 staining of tumor cells.¹⁵ This was confirmed by Barker et al who examined six cases of BCC.¹⁶ However, they demonstrated a more intensely ICAM-1 expression of dermal endothelial cells in areas of inflammatory infiltrate compared to normal skin. Groves et al showed variable staining patterns of endothelial E-selectin expression in a group of eleven BCCs.^{17,18} Finally, Pentel et al in a group of 16 BCCs and Viac et al in a group of five BCCs, described similar results.^{19,20} BCC tumor cells were all ICAM-1, E-selectin and VCAM-1 negative. Endothelial cells within the surrounding of the tumors weakly expressed ICAM-1 and rarely expressed E-selectin and VCAM-1.

In the present study, we investigated the expression and distribution of the adhesion molecules ICAM-1, VCAM-1 and E-selectin, together with their leukocyte receptors LFA-1, VLA-4 and CLA respectively, in thirty-three BCCs of different histological subtypes. We demonstrated moderate ICAM-1 and minimal VCAM-1 and E-selectin expression by endothelial cells of blood vessels surrounding BCC tumor lobules. This is in accordance with the staining results of their counter molecules, i.e. the demonstrated positivity for LFA-1 and the minimal staining for VLA-4 and CLA.

Both in nodular- and morpheaform type BCCs, expression of ICAM-1, E-selectin and VCAM-1 by tumor cells or overlying benign epidermal keratinocytes was absent.

Adhesion of lymphocytes to target cells via surface molecules is thought to be required for T-lymphocyte mediated immune reactions. It has been postulated that BCC can escape immunosurveillance because of the absence of adhesion molecules on tumor cells, preventing binding of T lymphocytes.^{9,16} This could explain the histological observation that T cells do not infiltrate BCC tumor lobules.

The inability of BCC tumor cells to express adhesion molecules is not absolute. Taylor et al presented a study where they demonstrated induction of ICAM-1 expression on tumor cells after BCC tissue had been incubated *in vitro* with IFN- γ .¹⁵ They put forward three possible explanations for the discrepancy between *in vivo* and *in vitro* observations: a) insufficient *in vivo* cytokine levels, b) a barrier preventing cytokines from reaching and interacting with tumor cells *in vivo*, and c) lower level of sensitivity.

In our opinion, the absence of adhesion molecules on BCC tumor cells as well on the overlying epidermal keratinocytes as demonstrated in this study, together with the moderate ICAM-1 and minimal VCAM-1 or E-selectin expression on the surrounding peritumoral endothelial cells could probably best be explained by insufficient *in vivo* cytokine levels. Insufficient *in vivo* cytokine levels can be caused by an insufficient amount of activated T-lymphocytes responding to antigen stimulation, or, as Yamamura et al demonstrated, an accumulation of type-2 instead of type-1 T-helper cells, resulting in the production of IL-4, IL-5, and IL-10 cytokines instead of the cytokines IL-2, IFN- γ and TNF- α .²¹⁻²³

In summary, endothelial cells are critical elements in the evolution of cellular infiltrates. Our results indicate "resting" endothelial cells around BCCs, with moderate ICAM-1 expression and minimal E-selectin/VCAM-1 expression, recruiting particularly LFA-1 positive lymphocytes in both nodular and morpheaform type BCC. Histologically, these LFA-1 positive lymphocytes do not infiltrate the BCC tumor lobules. This may be due to the absence of adhesion molecule expression on BCC tumor cells, interfering with the essential cell-cell contact between the effector immune cell and target cell.

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Chapter 3

Immunohistochemical localization of granzyme B in peritumoral infiltrates of basal cell carcinoma

M.E.J.M. Verhaegh, P.C. Wever,
H.A.M. Neumann, R. Hoekzema

Br J Dermatol 1997;137:1012-4 (correspondence)

Summary

BCCs are frequently associated with a peritumoral mono-nuclear cell infiltrate that may represent a specific antitumor response. The infiltrate largely consists of T cells with a predominance of the CD4+/T helper over the CD8+/T cytotoxic subset, together with small numbers of natural killer cells. Besides identifying the predominant cell phenotype, information about the activation status of lymphocytes also is essential. The presence of characteristic granules in the cytoplasm of cytotoxic T cells and natural killer cells correlates with activation and subsequent cytolytic potential of these cells *in vitro*. These cytoplasmic granules contain a pore-forming protein, perforin, and several homologous serine proteinases called granzymes. The action of granzymes results in the apoptotic death of the target cell. Recently, monoclonal antibodies became available, directed against a serine proteinase called granzyme B (grB). We investigated the expression of grB in 10 cases of BCC, of which 8 were untreated and 2 had been treated once with intralesional interferon- α 2b. (Double) staining with anti-CD3 or anti-CD8 antibodies was performed to identify the phenotype of grB positive cells. Immune infiltrates of BCC were compared with other skin tumors.

The results show that only a limited number of cytotoxic T cells surrounding BCC tumor lobules expressed grB. The small number of cells in the infiltrate that stained for grB mostly represented NK cells.

We conclude that in patients with BCC, the "activated" state of peritumoral T cells apparently does not lead to expression of granzymes and subsequent cytolytic activity by cytotoxic T cells present in these infiltrates.

Introduction

BCC represents the most common human malignancy in light-skinned individuals and accounts for at least 75% of all skin cancer.¹ Characteristically, BCC is a locally invasive tumor that grows slowly and rarely metastasizes. Increased numbers of lymphocytes surrounding BCC tumor lobules have been observed.² The infiltrate surrounding BCC tumor islands largely consists of T cells with a predominance of the CD4+/T-helper (Th) over the CD8+/T-cytotoxic (Tc) subset (Th/Tc ratio of 1.9 ± 0.8), similar to that found in typical delayed hypersensitivity reactions.³⁻⁴ Natural killer cells (NK) and B-cells are seen in much lower numbers.³⁻⁴

Several studies have shown expression of activation markers on 30-50% of T cells in BCC.⁵⁻⁷ Regression of BCC, either spontaneously or after local immunotherapy, correlates with increased numbers and activation state of peritumoral T cells, whereas aggressive tumor behaviour is associated with decreased numbers of T cells in peritumor areas.⁴ Although these data suggest a cell mediated anti-tumor response, the role of cells with cytolytic potential, i.e. Tc and NK cells in immune surveillance of BCC, is not clear.

Tc and NK cells are the effector cells in major histocompatibility complex (MHC)-restricted and in non-MHC-restricted cellular cytotoxicity, respectively.⁸ These cytotoxic reactions are important in the elimination of tumor cells and virus infected cells and for the process of allograft rejection.⁹

Our objective was to study the expression of grB by cells present in immune infiltrates surrounding BCC, as an indicator of cytolytic activity against the tumor. GrB is a serine proteinase stored in granules of activated Tc and NK cells and an unique activation marker of cytotoxic cells.¹⁰⁻¹² We investigated the expression of grB in 10 cases of BCC, of which 8 were untreated and 2 had been treated with a single intralesional injection of interferon (IFN)- α 2b. (Double) staining with anti-CD8 or anti-CD3 antibodies was performed to identify the phenotype of grB positive cells. Immune infiltrates of BCC were compared with those of squamous cell carcinoma (SCC), melanoma and inflamed seborrheic keratosis (SK). This is the first study on expression of grB in human skin cancer.

Methods

Tissue specimens

Formalin fixed, paraffin embedded biopsies or surgical specimens of non-ulcerating nodular-type BCC (n=8), SCC (n=5), melanoma (n=4) and SK (n=3), selected for the presence of moderate to marked peritumoral infiltrates, were ob-

tained from the files of the Department of Pathology, University Hospital Maastricht, The Netherlands.

Two cases of clinically diagnosed (non-biopsied) non-ulcerating nodular BCC, localized in the face, were treated by a single intralesional injection of 1.5×10^6 IE IFN- $\alpha 2b$, followed by excision after 48 hours.

Immunohistochemical staining

Serial paraffin sections, 4 μm thick, were placed on 3-aminopropyltriethoxysilane (Sigma, ST.Louis, USA) coated glass slides. Slides were deparaffinized and rehydrated and endogenous peroxidase activity was blocked by immersion for 20 min in methanol containing 0.3% H_2O_2 . Immunoreactivity was restored by boiling of slides in 10 mM sodium citrate (Merck, Darmstadt, Germany), pH 6.0, for 10 min, after which the slides were rinsed with tapwater, distilled water and phosphate buffered saline (PBS). Slides were stained at room temperature using the streptavidin-biotin horseradish peroxidase (HRP) complex technique. The staining sequence was: 10% normal goat serum in PBS for 15 min; monoclonal antibody (mAb) grB-7 or IgG_{2a} isotype control mAb in PBS (final concentration 2.5 $\mu g/mL$) for 1 hour; biotin-labeled rabbit anti-mouse F(ab')₂ fragments (DAKO, Glostrup, Denmark) in PBS containing 10% normal human serum (PBS-NHS) for 30 min; HRP-conjugated streptABComplex (DAKO) in PBS-NHS for 30 min. HRP activity was detected using 3,3-diaminobenzidine tetrahydrochloride as dye and H_2O_2 as substratum yielding a brown color. Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted.

For double staining of mAb grB7 with anti-CD3 polyclonal antibodies or anti-CD8 mAb, sequential staining techniques with alkaline phosphate- and HRP labelled secondary antibodies were used, respectively. CD3 or CD8 were detected using 3-amino-9-ethylcarbazole as the dye (red) and H_2O_2 as substratum. MAb grB7 was detected using naphtol-AS-MX phosphatate as substratum and Fast Blue BB as dye (blue).

Immunohistological evaluation

Cells displaying a strong cytoplasmic granular staining were considered grB+. GrB expression was scored by counting the number of positive cells per field in two representative areas by high power field magnification (400x) (0:0; +: 1-5; ++: 6-10; +++: 11-20; ++++: >20 positive cells per field). Double stained slides were used for phenotypic analysis of grB+ cells. Cells staining positive for grB as well as either CD3 or CD8 were considered Tc cells. Cells staining positive for grB only were considered NK cells.

Table I. Quantification of granzyme B positive cells per high power field magnification (400x)

Case	Tumor	grB+	grB+ Tc	Tumor invasion
1.	BCC	+	0	0
2.	BCC	+	0	0
3.	BCC	++	+	0
4.	BCC	+	0	0
5.	BCC	++	0	0
6.	BCC	0	0	0
7.	BCC	+	0	0
8.	BCC	+	0	0
9.	BCC + IFN- α 2b	+++	+	0
10.	BCC + IFN- α 2b	+++	+	0
11.	SCC	++++	+	++
12.	SCC	++++	+	++
13.	SCC	+++	+	+
14.	SCC	+++	+	+
15.	SCC	++++	++	+
16.	Melanoma	+++	+	+
17.	Melanoma	++	+	+
18.	Melanoma	++++	+	++
19.	Melanoma	+++	+	+
20.	SK	+++	+	++
21.	SK	+++	+	++
22.	SK	+++	+	+

(grB+: granzyme B positive cells, grB+ Tc: granzyme B positive CD8+/T-cytotoxic cells, Tumor invasion: invasion of granzyme B positive cells into the tumor lobules).

Results

A prominent lymphocytic infiltrate localized preferentially in perivascular areas and adjacent to and at the base of tumor nests was observed in all eight cases of untreated nodular BCC. Significant numbers of lymphocytes were not observed within the tumor.

To study the distribution and phenotype of grB+ cells in these infiltrates immunohistochemical single-colour and double-colour staining techniques were used. The results are summarized in Table I. Despite the presence of large numbers of CD3+ and CD8+ T cells, there was a striking absence of grB+ cells in peritumoral areas (Fig.1). The limited number of cells that expressed grB did not co-express CD3 or CD8 and consequently represented NK cells. GrB+ NK cells were scattered mainly throughout the peritumoral stroma, but did not infiltrate the tumor

Figure 1. BCC, single stained for grB. The peritumoral infiltrate does not show significant grB expression. Some grB+ cells (arrows) are observed inside or close to capillaries (x 200).

lobules. Relatively larger numbers of grB+ NK cells as well as sporadic grB+ Tc cells (defined as cells staining double positive for grB and either CD3 or CD8) were present inside or close to capillary lumina, distant from the tumor islands. In the two cases of BCC treated with IFN- α 2b 48 hours prior to resection, the number of grB+ cells in perivascular areas appeared to be increased in comparison with untreated BCC. Again these cells were mainly NK and not Tc cells. GrB+ cells adjacent to the tumor were, however, still sporadic (Fig.2a,b).

In contrast to BCC; SCC, melanoma and SK showed significant numbers of grB+ NK cells not only “crowding” directly adjacent to the tumor, but also invading tumor islands (Fig.3a,b). The number of grB+ Tc cells in these conditions was small, although increased compared to BCC.

The inability to detect significant numbers of grB+ Tc cells was not due to a technical problem, as grB+ Tc cells were easily detected in the same experiment in cytopsin preparations of peripheral blood lymphocytes from a healthy donor (not shown).

Figure 2a. BCC, 48 hours after a single intralesional injection with IFN- α 2b, doublestained for CD3 and grB (arrows). The number of peritumoral grB+ cells is still small (x 100).

Figure 2b. BCC, 48 hours after a single intralesional injection with IFN- α 2b, doublestained for CD3 and grB (arrows). Detail of a dermal capillary, close to an IFN- α 2b treated BCC. NK cells expressing grB but not CD3, as well as T cells that do not express grB are invading the dermis (x 320).

Discussion

In the last decade, several reports concerning the local immune response in BCC have attempted to elucidate the function of the cellular inflammatory infiltrate surrounding these skin cancers and their possible role in controlling tumor growth.^{13,14} The relatively benign tumor-behaviour, the areas of spontaneous regression and the therapeutic response to intralesional administration of cytokines, such as IL-2, IFN- γ and IFN- α , are in favour of a "specific" anti-tumor immune response in BCC. The fact that the stromal inflammatory reaction surrounding BCC is usually mild, that T cells do not touch or infiltrate BCC tumor lobules and that the frequency of BCC in immunosuppressed patients is only slightly increased compared with SCC, argue against a specific anti-tumor response.¹⁵ Different cells of the immune system have been implicated in tumor immunity, although their precise role remains unclear. It seems likely that helper T lymphocytes, lymphokine-secreting T lymphocytes, cytotoxic T lymphocytes, natural killer cells, macrophages and Langerhans cells each play some part in the process.¹⁵ Hunt et al showed a statistically significant increase of the number of T lymphocytes in actively regressing BCCs compared with non-regressing BCCs. This was caused by accumulation of CD4+ T cells, which are usually helper and/or cytokine-secreting lymphocytes, without any difference in CD8+ cells.¹⁴

Figure 3. In contrast with BCC, **(a)** single staining of SCC for grB shows a significant number of grB+ cells close to the tumor islands (x 200); **(b)** SCC, double stained for CD3 (grey) and grB (black). CD3-/grB+ NK cells invade the tumor. Double stained Tc cells are hardly present (x 100).

These results are consistent with those of Mozzanica et al who studied therapeutically induced regression of BCCs. After intralesional therapy with IFN- α 2b, an increased number of CD4+ T cells was observed within the tumor regions, thus confirming the importance of this cell type in mediating tumor regression.¹⁶

In this study, we investigated the possible role of CD8+ T cells and NK cells as other active participants in immune surveillance towards BCC.

Cytotoxic T cells (CD 8+) induce target cell death either by surface interaction between Fas ligand and Fas or by the release of perforin and granzymes.¹⁷ Both lytic pathways induce apoptosis. The perforin/granzyme pathway is a mechanism of cell-mediated cytotoxicity that is critical for the function of CD8+ lymphocytes and NK cells.¹⁸ After effector-target cell conjugation, the effector cell releases its cytolytic granules containing perforin and granzymes. Perforin undergoes polymerization in the presence of calcium and forms channels in the target cell membrane. The perforin-formed channels are postulated to allow the entry of granzymes into target cell cytoplasm. The action of granzymes results in the apoptotic death of the target cell.

Shresta et al clearly showed that grB is one of the molecules required for the induction of DNA fragmentation in target cells.¹⁸ The expression of grB can be used as a specific, functional marker for activated cytotoxic T cells *in vivo*, i.e. grB characterizes a specific T cell population that has been stimulated by recognition of its target. Only these T cells own lytic potential.

Until now, grB expression has been demonstrated in synovial tissue from patients with rheumatoid arthritis and osteoarthritis, in acute renal and cardiac allograft rejection and in Reed-Sternberg cells of Hodgkin's disease.¹⁹⁻²² Phenotypic analysis, in these studies, showed that expression of grB was restricted to CD56+ NK cells and CD3+ cells, representing cytotoxic T-lymphocytes.

To the best of our knowledge, our study represents the first report on expression of granzymes in immune infiltrates of skin cancer in man. Although we studied a limited number of tumors, we conclude that in BCC, in contrast with SCC, melanoma and "inflamed" SK, hardly any grB expression is present in peritumoral areas. In accordance with the relative paucity of NK cells in BCC,^{3,4} we detected a limited number of grB+ NK cells. Furthermore, we were unable to demonstrate significant numbers of Tc cells expressing grB in BCC, despite the presence of CD8+ T cells. It has previously been shown that there is a relative paucity of CD8+ T cells in the infiltrate surrounding BCC tumor islands.^{3,4} Here, we show that the cytotoxic potential of these cells, as reflected by expression of grB, also appears to be limited. It remains to be established if this lack of grB expression by CD8+ T cells in peritumoral areas is caused by local absence of lymphokines or by certain local (tumor-derived) immunosuppressive factors.

We analyzed 2 BCCs after a single dose of IFN- α 2b. After 48 hours increased numbers of grB+ NK cells as well as grB+ Tc cells appeared to be recruited from

the circulation. This recruitment may involve “vascular targeting”, mediated by cell adhesion molecules and may initiate tumor regression.²³ Yet, further studies are required to monitor cytotoxic events during local immunotherapy of BCC. In order to develop more efficient immuno-therapeutical strategies for BCC, future studies should focus more on the induction of granzymes or other cytolytic components by tumor-infiltrating Tc cells, as these cells are considered to be antigen-specific and therefore more effective in tumor cytotoxicity than NK cells. In conclusion, we investigated the functional role of cytotoxic lymphocytes in BCC, by analysis of grB expression. The majority of peritumoral Tc cells did not express grB, whereas only a limited number of grB+ NK cells was present. As grB is considered to be instrumental in cell-mediated cytotoxicity by cytotoxic lymphocytes, these results are indicative for a minor role of these cells in anti-tumor immune responses in BCC.

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Expression of the apoptosis-suppressing protein bcl-2 in non-melanoma skin cancer

M.E.J.M.Verhaegh, C.J.G.Sanders,
J.W.Arends, H.A.M.Neumann

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Summary

Basal cell carcinoma (BCC) is a typically slow growing malignant tumor, composed of cells similar to those in the basal area of the epidermis. We investigated the expression of *bcl-2* (B-cell leukemia/lymphoma-2) in BCC and also in squamous cell carcinoma (SCC) of the skin. The proto-oncogen *bcl-2* encodes an inner mitochondrial membrane protein that inhibits programmed cell death (apoptosis). The protein is expressed in basal cells in normal human epithelium, but not in the suprabasal cell layers. Immunohistochemical localization using a monoclonal anti-*bcl-2* antibody revealed positive *bcl-2* expression of all BCCs (15 patients). SCCs failed to express *bcl-2* (5 patients). The positive *bcl-2* staining of BCC tumor cells supports the hypothesis that BCCs originate from the basal layer of the epidermis. The *bcl-2* expression of BCC tumor cells also suggests a neoplastic transformation caused by extended cell survival instead of cell proliferation. This kind of neoplastic growth is possibly associated with less aggressive tumor behaviour.

Introduction

BCC and SCC of the skin are malignant neoplasias that originate from epidermal keratinocytes. Both predominantly develop on sun-exposed sites either as a result of UV-induced DNA damage or UV-induced immunosuppression.¹ BCCs are typically slow growing tumors, often taking months to years to reach significant proportions.² Several explanations for this clinical behaviour have been proposed: 1) tumoral prolongation of the S-(DNA synthesis) phase of the cell cycle;³ 2) a small percentage of tumor cells that are actively proliferating;⁴ 3) tumor regression in response to host immune factors;⁵ 4) limited enzyme lysis of adjacent connective tissue;⁶ 5) mechanical resistance of the dermis counteracting the expansion of the carcinoma.⁶ Kerr et al, describing the phenomenon of apoptosis (self-destruction of single cells), suggested that prominent cell death in BCC may provide an alternative explanation for their clinically slow growth rate.⁷

A fundamental feature of carcinogenesis is deregulation of normal cellular genes termed proto-oncogenes.⁸ The products of activated ras oncogenes and mutated tumor suppressor genes act to cause increased cellular proliferation.⁹

Recently however, a new class of proto-oncogenes has been defined, which contributes to malignancy by inhibiting programmed cell death.¹⁰ *Bcl-2* (B-cell leukemia/lymphoma-2) proto-oncogene is the first member of this class of oncogenes, and was originally discovered at the chromosomal translocation point t(14;18) in a large proportion of follicular lymphomas.¹¹ *Bcl-2* shows the unique functional role of blocking programmed cell death, referred to as apoptosis, independent of affecting proliferation. Studying normal adult skin, Lu et al found *bcl-2* expression in the basal cell layer and the upper third of mature hair follicles but not in the suprabasal cell layers of the epidermis.¹² Since BCC is thought to arise from basal keratinocytes, *bcl-2* expression is expected on BCC tumor cells. On the other hand the described prominent cell death in BCC^{7,13} would imply an absence of *bcl-2* expression. Squamous cell carcinoma that originates from suprabasal keratinocytes would be expected to be negative for *bcl-2*.

The present study was performed to identify the expression of *bcl-2* in normal skin, BCC and SCC.

Material and methods

Tissue specimens

Fresh frozen skin samples were obtained from patients undergoing Mohs' micrographic surgery for BCC (15 patients) and SCC (5 patients), excised from sun-exposed areas of the head. The BCCs consisted of 7 with morphea-like and 8 with solid-type histopathological growth-patterns. Frozen samples were stored at

Figure 1. Immunohistochemical analysis of cryostat sections of BCC with prominent homogeneous cytoplasmic *bcl-2* expression. **(a)** Solid-type 90x, **(b)** Morphea-type 200x.

-70°C. Normal skin from various body sites was obtained from the tips of elliptical skin biopsies of dermal naevi and processed in the same way. Five formalin-fixed specimens of BCC were also included.

Immunohistochemical staining

Frozen sections of 5µm thickness were cut, placed on gelatine-alum coated slides, and fixed in acetone at 4°C for 10 min. After rinsing with PBS, slides were incubated with primary antibody (see below). Sections of 3 µm were cut from routinely processed specimens (fixed in 4% buffered formaldehyde overnight at room temperature, embedded in paraffin wax), placed on slides coated with gelatine-alum, and dried overnight at 56 °C. The specimens were subsequently dewaxed in xylene and dehydrated in absolute ethanol. Endogenous peroxidase was blocked with 3% H₂O₂ in absolute methanol at room temperature (RT). Slides were rehydrated in decreasing concentrations of ethanol, and rinsed in distilled water. Subsequently, the slides were placed in jars containing 250cc 10 mmol/l citrate in distilled water, pH=6, placed in a conventional microwave oven, microwaved until boiling, and then microwaved for another 10 min. The jar was then removed

Figure 2. Immunohistochemical analysis of paraffin sections of solid-type basal cell carcinoma with homogeneous cytoplasmic *bcl-2* expression. (a) 40x, (b) 140x.

from the microwave oven, and left to cool for 20 min until RT was achieved. Slides were rinsed in PBS.

The slides (both frozen sections and microwave treated) were then incubated with the monoclonal antibody *bcl-2* (clone 124, purchased from DAKO, Denmark) for 45 min at RT. A biotin-streptavidine detection system was employed with DAB (Sigma) as the chromogen. Slides were washed repeatedly with PBS, and incubated with biotinylated rabbit-anti-mouse for 30 min. After rinsing in PBS, slides were incubated with the peroxidase-conjugated streptavidin label (DAKO) for 30 min then rinsed in PBS, and subsequently incubated with DAB/Imidazol (1:1) for 5 min. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted with Entellan. Normal skin sections served as positive controls. Negative controls were treated as above with exception of the *bcl-2* antibody incubation step.

Results

Twenty specimens from 15 patients with BCC (15 frozen-samples, 5 formalin-samples), five SCCs (frozen-samples) and five normal skin specimens (frozen-samples) were assessed for *bcl-2* expression by immunohistochemical staining. A fine granular brown cytoplasmatic staining of the cells with *bcl-2* was considered positive. In the epidermis of normal skin *bcl-2* expression was uniformly present in the basal cell layer. Epithelial cells in the infundibulum of hair follicles also showed expression of *bcl-2*, as well as cells lining the duct of sebaceous glands and eccrine sweat glands. The suprabasal layers were consistently negative. All BCCs were *bcl-2* positive with prominent fine granular cytoplasmic staining throughout the lesions. There were no staining differences between frozen (Fig. 1a,b) and formalin (Fig. 2a,b) fixed materials or between solid and morphea-type growth-patterns of BCC. SCCs failed to express *bcl-2*.

Discussion

Both in normal tissue development and malignant cell growth, the maintenance of cell numbers reflects a balance between cell proliferation and cell death. Excessive growth may result from uncontrolled cellular proliferation as well as from limited cell death. To date, most of our knowledge concerning oncogenic events has concentrated on mechanisms of increased cellular proliferation. Only recently attention has focused on the possible consequences of abnormal or defective cell death.¹⁴

Apoptosis is one of two forms of cell death that have been described in mammalian tissue, the other being the pathological process of necrosis. Apoptotic cells characteristically show a decrease in volume, clumping of chromatin and nuclear fragmentation, and blebbing of the cell membrane forming apoptotic bodies.¹⁵ This mode of physiological cell deletion appears to be intrinsically programmed, and serves as a regulator of tissue size. The blocking of this programmed cell death is attributed to the gene products of the *bcl-2* (B-cell leukemia/lymphoma-2) proto-oncogene. *Bcl-2* is localized at the inner site of mitochondrial membranes and is expressed in lymphoid cells and several epithelial tissues (skin and intestine) where it is detectable in basal cells but not in more superficial differentiated cells.¹⁶ It has been suggested that this pattern of expression assists the survival of stem cells while preventing the overaccumulation of differentiated cells.¹⁷

Overproduction of the *bcl-2* protein in transgenic mice provided evidence for the tumorigenic potential of these products in that they prevented cell death, demonstrated by prolonged cell survival and accumulation of cells.¹⁸

BCC is a malignant tumor, which rarely metastasizes, composed of cells similar to those in the basal cell layer of the epidermis and its appendages.¹⁹ Prominent cell death is suggested as an explanation for the clinically slow growth rate.^{7,13,19} Our immunohistochemical study of BCC using a monoclonal anti-*bcl-2* antibody revealed that all tumors (15 frozen, 5 formalin samples) were *bcl-2* positive which would support the hypothesis that BCCs originate from cells from the basal layer of the epidermis. The prominent *bcl-2* expression of BCC tumor cells suggests also that inhibition of programmed cell death occurs, and would argue against the suggestion that prominent cell death in BCCs accounts for their typically slow growth rate.

Possibly a BCC is a neoplastic transformation caused by cell survival, independent of cell proliferation. This hypothesis is supported by the following results. Tritiated thymidine labeling studies suggest that only a small percentage of the total tumor volume is actively proliferating.⁴ A study with the proliferation-associated Ki-67 antigen displayed Ki-67 reactivity either to the nuclei of three to five rows of peripheral cells, or to nuclei scattered in the central as well as in the peripheral parts of the tumor strands.²⁰ Activated ras-oncogenes, which are related to increased cellular proliferation, are rare events in human non-melanoma skin cancer and it seems unlikely that mutational activation of ras genes is a common feature responsible for the induction of BCC.²¹ In BCC an aberrant expression of *p53* protein has been reported.²² The wild type *p53* gene is located on chromosome 17p and functions as a tumor suppressor gene by acting like a brake on tumor growth to allow optimal repair of damaged DNA. It is suggested that if this repair is not successful the *p53* gene induces apoptosis. The mutant version of *p53* is possibly acting similar to *bcl-2* in blocking apoptosis.²³

It is interesting to speculate that BCC is a result of increased cell survival, since some authors suggest that inhibition of programmed cell death may lead to neoplastic growth at slower rates than found with growth induced by oncogenes that act by stimulating cell proliferation.²⁴

SCC is a malignant tumor arising from the suprabasal keratinocytes of the epidermis and its cells show variable degrees of maturation and keratin formation. As mentioned before *bcl-2* is detectable in the basal cells of the epidermis, but not in the suprabasally located differentiated keratinocytes. There is evidence that in SCC in-situ of the skin increased cell proliferation is present.²⁵ Our findings of absent *bcl-2* staining in SCC of the skin suggest that increased proliferation rather than decreased cell death attributes to tumorigenesis in this type of skin cancer.

In conclusion, the expression of the apoptosis-suppressing protein *bcl-2* together with the absence of activated ras-oncogenes related to cell proliferation and the small percentage of actively proliferating tumor cells, support the concept that BCC is a neoplastic transformation caused by extended cell survival. Neoplastic

growth as a consequence of extended cell survival instead of cell proliferation could possibly be a new explanation for the typical slow growth rate of BCC.

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Transforming growth factor-beta and bcl-2 distribution patterns distinguish trichoepithelioma from basal cell carcinoma

M.E.J.M. Verhaegh, J.W. Arends, I.M.L. Majoie,
R. Hoekzema, H.A.M. Neumann

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Summary

Trichoepithelioma (TE) and basal cell carcinoma (BCC) have many features in common both clinically and histologically. Despite these many similarities TE and BCC represent different biological entities.

Recently, *bcl-2* and *CD34* have been reported as reliable markers in distinguishing the two types of tumor. *TGF- β* , a multifunctional regulator of both cell growth and differentiation, was evaluated in this study. The immunohistochemical expression of *TGF- β* was compared with the distribution patterns of *bcl-2* and *CD34* in 5 BCCs, 5 TEs and 7 borderline cases. All 5 TEs showed a diffuse cytoplasmic staining of tumor cells for *TGF- β* , whereas 4 of 5 BCCs were *TGF- β* negative. Of the 7 equivocal cases of TE/BCC, 5 tumors demonstrated *TGF- β* positivity in combination with negative *bcl-2* staining corresponding to TE. The remaining 2 cases demonstrated the opposite staining pattern, characteristic for BCC.

The *TGF- β* staining pattern appears to be a helpful additional marker together with *bcl-2* in differentiating between trichoepithelioma and BCC. The demonstrated staining differences may relate to the distinct origin and biological behaviour of the two tumors and may therefore be of value in subsequent patient management.

Introduction

Trichoepitheliomas (TE) are benign tumors confined to the dermis and characterized by aggregates of neoplastic epithelial cells with limited follicular differentiation.¹ Clinically, lesions appear as firm, skin colored nodules or papules mostly located on the face. Large lesions may be traversed by telangiectasias and sometimes ulcerate. This type of TE can be mistaken clinically for BCC. The histological distinction between TE and BCC also can be a difficult diagnostic dilemma, especially for the infundibulocystic type of BCC.² This distinction is important because of the consequences for subsequent patient management. BCC displays a much more aggressive tumor behaviour than TE, and should be excised completely.

Recently, *bcl-2* (B-cell leukemia/lymphoma-2) and *CD34* expression have been reported to be of diagnostic help in differentiating between TE and BCC.^{3,4} *Bcl-2* functions as a repressor of programmed cell death, and is described to be present in BCC but absent in TE by immunohistochemical analysis. *CD34* expression was demonstrated in the peri-tumoral stromal cells of TE, whereas the adjacent stroma of BCC stained negative. In addition to *bcl-2* and *CD34*, we selected *TGF-β* (Transforming Growth Factor-β) because of its pleiotropic effects on cellular differentiation and growth.

17 cases of TE/BCC were studied and the results of the hematoxylin-eosin stained histological sections compared with immunohistochemical analysis of consecutive sections stained for *TGF-β*, *bcl-2* and *CD34*.

The aim of this study was to find an additional diagnostic tool to discriminate trichoepithelioma from BCC. The possible relation of our findings to the different biological characteristics of the two entities is discussed.

Material and methods

5 cases of unequivocal TE and 5 of nodular type BCC diagnosed histologically by the hospital pathologist in charge were obtained from the files of the department of Pathology, Academic Hospital Maastricht. An additional 7 borderline cases, i.e. tumors in which reliable discrimination between TE and BCC on routine histologic grounds was found to be difficult by the pathologist in charge, were selected too. Next, the total of 17 cases of TE and/or BCC were reviewed randomly by three dermatopathologists (A.V., M.D., R.H.). 10 cases were unequivocally diagnosed as BCC (n=5) or TE (n=5) by all three pathologists. The remaining 7 cases, corresponding to the original 7 borderline cases, were equivocally classified as either BCC or TE. Histologic criteria included the presence or absence of epidermal connections, ulceration, significant inflammatory response, tumor-stromal clefts,

Table I. Clinical features of 5 BCCs, 5 TEs, and 7 borderline cases

Pat no.	Age	Sex	Site	Remarks
1.	82	M	back	
2.	67	M	temple	
3.	64	M	temple	BCC 4x
4.	49	F	nose	
5.	45	F	forehead	BCC 2x, SCC 8x
6.	53	F	neck	
7.	59	F	nose	
8.	59	F	upper lip	BCC 3x
9.	51	M	eyebrow	
10.	67	M	nose	
11.	33	M	forehead	
12.	59	F	cheek	
13.	50	F	eyelid	Melanoma
14.	51	F	nose	
15.	67	M	eyelid	BCC 2x
16.	76	F	nose	BCC 3x
17.	69	F	chin	

SCC: Squamous cell carcinoma

Table II. Incidence of histologic features of the unequivocal 5 BCCs (tumor no. 1-5) and 5 TEs (tumor no. 6-10), and the 7 equivocal cases of BCC/TE (tumor no. 11-17), all classified on morphological grounds. Followed by the classified 5 TEs (tumor no. 11-15) and 2 BCCs (tumor no. 16-17) by immunohistochemical analysis of the equivocal group.

Feature	BCC (%) N=5	TE (%) N=5	BCC/TE (%) N=7	TE (%) N=5	BCC (%) N=2
Epidermal connections	80	27	43	60	0
Tumor-stromal clefts	80	0	48	47	50
Inflammatory response	80	0	29	20	50
Mitotic figures	60	47	27	20	17
Fibrotic stroma	27	100	62	67	33
Infundibular cysts	13	87	67	60	66
Follicular differentiation	7	93	52	47	66

Table III. Results of the routine histological sections (diagnoses of the three dermatopathologists on morphological grounds), immunohistochemical analysis and final diagnosis of 5 BCCs, 5 TEs, and 7 equivocal cases of BCC/TE.

Tumor no.	Routine histology	TGF- β	BCL-2	CD34	Final Diagnosis
1	BCC-BCC-BCC	-	+	+	BCC
2	BCC-BCC-BCC	-	+	+	BCC
3	BCC-BCC-BCC	-	+	-	BCC
4	BCC-BCC-BCC	-	+	-	BCC
5	BCC-BCC-BCC	+	+	-	BCC
6	TE-TE-TE	+	+	+	TE
7	TE-TE-TE	+	-	+	TE
8	TE-TE-TE	+	-	+	TE
9	TE-TE-TE	-/+	+/-	-/+	BCC
10	TE-TE-TE	+	-	ND	TE
11	BCC-TE-TE	+	-	+	TE
12	BCC-TE-TE	+	-	+	TE
13	BCC-TE-BCC	+	-	+	TE
14	BCC-TE-TE	+	-	ND	TE
15	BCC-TE-BCC	+	-	+	TE
16	BCC-TE-BCC	-	+	-	BCC
17	TE-TE-BCC	-	+	-	BCC

ND: not done.

mitotic figures, fibrotic stroma, infundibular cysts and follicular differentiation in the manner of follicular germs and papillae. After review of routine histologic sections additional 5 μ m-paraffin sections were cut and immunohistochemical analysis was performed essentially as described previously, using an avidin-biotin-peroxidase technique with 3,3' diaminobenzidine (DAB) as the chromogen and 1% haematoxylin as the counterstain.^{3,9,13} Primary antibodies were the following: *bcl-2* (clone 124, DAKO, Glostrup, Denmark), *CD34* (anti-HPCA-1, Becton Dickinson, Erembodegem-Aalst, Belgium) and *TGF- β* (code 1835-01; anti-*TGF- β* ₁, - β ₂, - β ₃, Genzyme, Cambridge, USA). Negative controls were treated as above, omitting the primary antibody incubation step.

Results

The clinical features of this series of TE/BCC are outlined in Table I. The patients (10 females, 7 males) ranged in age from 33-82 years (mean 57). All lesions except one were located in the head-neck region and existed for at least one year without

Figure 1a. Tumor no.7 demonstrating positive cytoplasmic staining of tumor cells for *TGF-β*, characteristic of TE. (magnification x 75).

Figure 1b. Tumor no.7 with positive *bcl-2* staining only at the periphery of tumor nests, characteristic of TE. (magnification x 75).

recent changes. All tumors were solitary except case no.6. The therapy of choice was excision in all cases. The duration of follow up ranged from 2-4 years. Until now, no recurrences of tumor have occurred. Six patients either had a history of skin cancer, or developed skin cancer at other sites afterwards.

The morphologic features of the 5 BCCs, 5 TEs and 7 equivocal cases of TE/BCC reviewed by three different dermatopathologists are compared in Table II. Column 4 and 5 contain the histological features of the 7 borderline cases after their classification as either TE (n=5) or BCC (n=2) by immunohistochemistry. The immunohistochemical results are presented in Table III.

TGF-β: A diffuse homogeneous cytoplasmic staining of tumor cells throughout the tumor nests was considered positive (Fig.1a). All 5 TEs, 1 BCC and 5 equivocal cases were *TGF-β* positive. Tumor no.9, diagnosed as a TE on routine histologic grounds, displayed a dual staining pattern with positive and negative areas in the same tumor lobules (Fig.2a). In this particular tumor *TGF-β* expression was restricted to areas of follicular differentiation and infundibulocystic structures. The remaining cases were unreactive for *TGF-β* or showed faint staining of spindle/squamoid cells in the centre of some tumor islands (Fig.3a). The peritumoral

Figure 2a. Tumor no.9 with markedly positive *TGF- β* staining of areas of follicular differentiation and infundibulo-cystic structures. (magnification x 75).

Figure 2b. Tumor no.9 with positive *bcl-2* staining of tumor cells throughout the tumor nests except areas of follicular differentiation and infundibulo-cystic structures. (magnification x 75).

stroma of both BCC and TE was largely negative. Positive staining was also noted in the cytoplasm of suprabasal normal keratinocytes and in endothelial cells.

Bcl-2: A fine granular cytoplasmic staining of tumor cells throughout the tumor nests with *bcl-2* was considered positive (Fig.3b). All 5 BCCs, 2 TE's and 2 equivocal cases were *bcl-2* positive. In contrast with *TGF- β* staining, tumor no.9 showed positive homogenous cytoplasmic *bcl-2* staining of tumor cells throughout the tumor nests except for the areas of follicular differentiation and infundibulo-cystic structures (Fig.2b). Tumor no.6, also diagnosed as a TE, displayed positive *bcl-2* staining of all tumor cells, but also of the entire epidermis and

Figure 3a. Tumor no.16 with negative *TGF- β* staining of tumor cells, characteristic of BCC. (magnification x 75).

Figure 3b. Tumor no.16 demonstrating prominent homogeneous cytoplasmic *bcl-2* expression of tumor cells, characteristic of BCC. (magnification x 75).

follicular structures. In the remaining cases staining was limited to the outermost layer of the proliferations (Fig. 1b). As in normal dermis, a few dermal perivascular lymphocytes were present in all cases of TE. These lymphocytes as well as the lymphocytic infiltrates surrounding BBCs together with basal keratinocytes of the epidermis served as internal positive controls for the antibody against *bcl-2*.

CD34: Expression of the *CD34* antigen by the mesenchymal cells adjacent to the tumor islands was considered positive. 3 TE's and 4 equivocal cases were *CD34* positive. 2 BCCs (tumor no.1 and 2) and one TE (tumor no.9) displayed staining in one area and absence of staining in another. In the remaining cases there was a

buffer zone of negatively stained cells separating the epithelial islands from the CD34-positive mesenchymal cells. In all sections there was CD34 positivity of endothelial cells.

Discussion

Precise differentiation between TE and BCC is important since their treatment and prognosis are different. Ulceration, tumor-stromal clefts, inflammatory response and mitosis are histologic features of BCC, whereas papillary mesenchymal bodies, fibrotic stroma, infundibulo-cystic structures containing cornified cells, and follicular differentiation in the form of germs and papillae are considered characteristic of TE.^{5,6} In general, the assessment of histologic features in 5 BCCs and 5 TEs by our panel of dermatopathologists, as depicted in Table I, proved to be in accordance with the above-mentioned diagnostic criteria.

Unfortunately the group of equivocal cases of BCC/TE often displays an inconsistent distribution of these features as demonstrated in column 3 of Table I. In these cases a clear diagnosis cannot be made in spite of detailed clinical information and routine histopathology. This observation is supported by the data in column 4 and 5 of Table II. After classifying the 7 borderline cases based on immunohistochemistry the 5 TEs and 2 BCCs still display a non-specific pattern of histological features.

Recently *bcl-2* and CD34 were introduced as helpful and reliable immunohistochemical markers to distinguish TE from BCC.^{3,4} The proto-oncogene *bcl-2* encodes a protein which inhibits programmed cell death (apoptosis), and is expressed in normal basal keratinocytes but not in the superficial layers.^{7,8} *Bcl-2* expression has been demonstrated intracellularly in a wide variety of neoplasms, including BCC.⁹ Smoller et al, who examined 10 BCCs and 10 TEs for *bcl-2*, found all BCCs positive with prominent homogenous cytoplasmic staining throughout the lesions, whereas in TEs, without exception, staining of tumor cells was limited to the outermost basaloid cells.⁴ Although we were able to confirm these results in general, we cannot explain the strong diffuse staining for *bcl-2* in tumor no.6, diagnosed as TE.

CD34, a 115-kd glycoprotein, is present in mesenchymal cells located around the middle portion of normal hair follicles.^{10,11} Since TEs are neoplasms with differentiation toward follicular epithelium, positive stroma staining around TE tumor nests was expected. Kirchmann et al observed strong peritumoral staining of CD34 in 16 TEs, but not in 19 BCCs.³ However, in our hands two out of five BCCs (tumors no. 1,2) displayed strong CD34 positivity of the immediate tumor stroma.

TGF-β is a multifunctional regulator of both cell growth and differentiation and has been shown to inhibit the growth of epithelial cells, stimulate mesenchymal cells and promote epithelial cell differentiation.¹² Our results of *TGF-β* staining in normal epidermis and in BCC tumor islands are in agreement with those of Stamp et al.¹³ However, we did not observe the abundant extracellular presence of *TGF-β* in peritumoral stroma, reported by these authors in the sclerosing types of BCC. This is unlikely to be caused by our use of a different antibody, as our antibody recognizes both precursor (intracellular) and activated (extracellular) *TGF-β*1 and *TGF-β*2. Maybe it is due to the fact that we studied only nodular and not sclerosing BCC.

To our knowledge the expression of *TGF-β* by TE tumor cells presented in our study, has not been reported before. The combination of staining for *TGF-β* and *bcl-2* seems to be valuable in differentiating this neoplasm from BCC. In general, TE displayed *TGF-β* positivity combined with negative *bcl-2* staining whereas BCC expressed the opposite. This enabled us to classify the 7 equivocal cases of TE\BCC (Table 2).

Glick et al studied *TGF-β*1 and *TGF-β*2 expression in murine skin and skin tumors.¹⁴ They showed that in the mouse *TGF-β* expression and response are compartmentalized, and that loss of *TGF-β* in epidermis is linked to increased risk for malignant conversion. As discussed by these authors, loss of expression of *TGF-β* from epidermal cells may be associated with increased proliferation and decreased apoptosis. If we extrapolate this to the human situation in BCC, loss of *TGF-β* expression could be a second mechanism responsible for extended cell survival besides expression of *bcl-2*. Moreover, the absence of *TGF-β* expression in BCC could be correlated with the apparent failure of the tumor cells to differentiate.

One could speculate that the *bcl-2* positive/*TGF-β* negative cells in BCC are responsible for the biologically more aggressive behaviour of these tumors, compared to the *bcl-2* negative/*TGF-β* positive cells in TE. So, tumors with a *bcl-2* positive/*TGF-β* negative staining pattern possibly deserve a more aggressive therapeutic approach.

The staining differences of *TGF-β* and *bcl-2* in TE and BCC together with their possible relevance for tumor behaviour, are well demonstrated in tumor no.9. On routine histologic grounds three dermato-pathologists diagnosed this tumor as a TE, because of features of follicular differentiation, cornification, fibrotic stroma and absence of clefts and inflammatory cells. In retrospect, we believe tumor no.9 represents an infundibulocystic BCC. This variant of BCC was described in 1990 by Walsh and Ackerman and recently discussed by De Eusebio et al.^{1,15} In general, the characteristic features of infundibulocystic BCC they reported are in accordance with our tumor no.9: (1) continuity of the neoplastic cells with infundibula, (2)

scant stroma, (3) follicular germs devoid of associated follicular papillae, (4) lack of differentiation toward outer sheath, inner sheath, or hair and (5) presence of neoplastic cells in mitosis. So, based on morphological grounds tumor no.9 can be diagnosed as an infundibulocystic BCC and not a TE. This is in agreement with the observation that most of the tumor cells stained *bcl-2* positive/*TGF-β* negative, except for areas of follicular differentiation and infundibulo-cystic structures, in which the opposite staining pattern was observed. More cases of neoplasms with follicular differentiation and a differential diagnosis of TE versus infundibulocystic BCC have to be studied, in order to determine the exact additional value of immunohistochemistry over morphological features in these particular cases. In conclusion, the clinical and histological distinction between TE and BCC sometimes proves to be a diagnostic dilemma. Staining for *TGF-β*, especially in combination with *bcl-2*, appears to be of additional but not final value in differentiating both tumors.

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Clinical and histological features of recurrent extensive basal cell carcinoma

M.E.J.M. Verhaegh, F.W.G. Grintjens, G.A.M. Krekels,
A.H.M. Vermeulen, H.A.M. Neumann

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Summary

Most basal cell carcinomas (BCCs) are small, slow growing, well defined lesions easily treated by various methods. On the other hand, there is also a group of aggressive BCCs characterized by extensive local invasion and disfigurement.

Possible risk factors, both clinically and histologically, were evaluated in a retrospective study of 72 recurrent extensive BCCs. Most BCCs were initially treated by surgical excision. The recurrent tumors were treated by means of Mohs' micrographic surgery.

The mean preoperative tumor size in our study was 2.1 cm. All 72 tumors except one were located in the face, of which the nasal-perinasal area was the most common site (51%). In 33% of the cases the histologic pattern displayed a mixture of solid and infiltrative components in the same tumor, 38% demonstrated a strictly nodular tumor growth, whereas 21% was of the infiltrative type.

Analysis of 72 recurrent extensive BCCs demonstrated the following characteristics: mean lesion diameter >2cm and tumor location in the central H zone of the face. Besides confirming the documented aggressiveness of the morpheaform type BCC, we also demonstrated extensive tumor growth of both nodular and nodular-infiltrative type BCC. The perinasal area in particular was characterized by extensive vertical tumor expansion of the nodular/nodular-infiltrative tumor type.

Introduction

BCC is the most common malignancy in man, predominantly found on sun-exposed areas. The incidence of BCC is rapidly increasing worldwide. In the Netherlands (15 million inhabitants), approximately 18.000 BCCs were diagnosed in 1994, with an expected increase of 2.700 BCCs yearly.¹

BCC can be categorized into subtypes morphologically and histologically. A convenient clinical classification includes superficial, nodulo-ulcerative, morphea-like or fibrosing, and pigmented types. In large studies, 45-60 percent are nodulo-ulcerative, 15-35 percent superficial, 1-2 percent pigmented, and the remainder 4 to 17 percent morpheaform.²

Histologically BCCs can be divided into two groups: undifferentiated and differentiated tumors. Keratotic BCC and adenoid BCC are examples of the latter group. BCCs showing no differentiation can be subdivided into multifocal superficial, nodular, micronodular, infiltrating (nonsclerosing vs. sclerosing), basosquamous and pigmented type.³

Most BCCs are slow growing, relatively non-aggressive tumors that are treated by various methods of treatment like conventional surgery, curettage and electro-desiccation, cryotherapy and radiotherapy. A minority of tumors, however, displays a more aggressive biological behaviour, with extensive local tissue destruction, multiple recurrences or, in rare cases, even metastasis.

Aggressive-growth BCC (AG-BCC) has been variously defined and studied previously by other investigators.^{4,5} AG-BCCs are a group of clinically and histologically aggressive tumors. This group includes infiltrating and recurrent BCCs. Other general criteria used to identify AG-BCC include, (1) patients under 35 years of age, especially women, (2) BCCs localized in the center of the face, and (3) increasing lesion diameter (>2cm).⁶⁻⁸ Inadequate treatment of AG-BCC will ultimately lead to recurrence and reconstructive challenges that possibly could have been avoided by recognizing this type of tumor initially.

Mohs' micrographic surgery (MMS), a method that provides complete microscopic control of the excision, seems particularly useful for managing these AG-BCCs.

MMS is a standard routine treatment for different types of BCC in the United States of America.⁹ In Europe, where MMS is less widely available, only AG-BCCs are treated by this technique.

In this study we retrospectively evaluated a selected group of recurrent extensive BCCs treated by MMS, in order to determine their clinical and histological parameters.

Patients and methods

Of a total of 371 cases of BCCs treated by MMS in the period 1992 -1996 at the dermatology department of the University Hospital Maastricht, 72 cases were selected. All selected cases were recurrent tumors. A recurrence was defined as the reappearance of a BCC within or contiguous to the scar resulting from the initial attempt at definitive treatment. The previous treatment modalities utilized for the recurrent tumors are summarized in Table I, categorized by tumor type. Forty-two (58%) recurrent tumors had been previously treated once, fifteen tumors (21%) twice, seven tumors (10%) three times, three tumors (4%) four times and five tumors (7%) were previously treated at least five times.

Besides the recurrence status, we also selected only those tumors that required at least three surgical Mohs stages before complete tumor removal was achieved. The minimum of three Mohs layers was chosen because of our specific interest regarding the histologic growth types of BCC and their subclinical horizontal and/or vertical extension pattern.

Next, all Mohs slides of each tumor were reviewed by at least two authors of this article. The histologic patterns of the BCCs were classified as follows: 1) nodular type: circumscribed, well-defined islands of tumor cells in the dermis with typically prominent peripheral palisading and retraction clefts, 2) infiltrative type: irregular tumor islands predominantly arranged as elongated strands with spiking contours, with (infiltrative morphea-form BCC) or without (infiltrative non-morphea-form BCC) fibrotic stromal response, 3) adenoid type: thin strands of basaloid cells in a reticulate pattern, surrounded by a mucinous stroma, 4) micronodular type: dispersed growth pattern with small nests without typically prominent peripheral palisading and retraction spaces, and 5) basosquamous type: composed of three types of cells: basaloid cells, squamoid cells and intermediate cells which resemble those seen in metatypical tumors.¹⁰ All cases were classified according to the direction of subclinical tumorspread by evaluating the Mohs mapping charts, in a prominent horizontal, a prominent vertical or an equal horizontal and vertical growth pattern, as demonstrated by Breuninger et al.¹¹

The histologic subtypes and the pattern of subclinical horizontal and vertical extension were correlated with age (at the moment of operation) and sex of the patient, size of the tumor before surgery (largest diameter in cm), size of the final defect (largest diameter in cm), and the anatomic location of the tumor (according to the specific site codes of the New York University Medical Center, University Hospital, oncology section).

Table I. The various histological BCC-types associated with their previous treatment modality.

BCC - type	Previous treatment	No.
Nodular - type (n=27)	surgery	14
	surgery / X-ray therapy	6
	cryotherapy	3
	surgery / cryotherapy	2
	surgery / X-ray / cryotherapy	1
	curettage-electrodesiccation	1
Infiltrative - type (n=15)	surgery	8
	X-ray therapy	2
	cryotherapy	1
	Mohs' micrographic surgery	1
	unknown	3
Nodular / Infiltrative - type (n=24)	cryotherapy	6
	surgery	5
	surgery / cryotherapy	4
	surgery / X-ray therapy	2
	X-ray therapy	1
	curettage-electrodesiccation	1
	Mohs' micrographic surgery	1
	surgery / X-ray / cryotherapy	1
	unknown	3
Micronodular - type (n=3)	surgery	2
	cryotherapy	1
Adenoid - type (n=2)	surgery	2
Basosquamous - type (n=1)	Mohs' micrographic surgery	1

Results

Seventy-two recurrent BCCs were reevaluated. All selected tumors required at least three surgical stages of MMS. The number of surgical Mohs stages and frozen slides was 3-7 (mean 4.1) and 4-55 (mean 17.5) respectively.

Forty-four (61%) of the patients were male between the ages of 43 and 86 years (mean, 68.2) and 28 (39%) were female between 33 and 94 years old (mean, 66.9). The overall mean age was 67.7 years (Fig.1).

Of 56 (78%) tumors pre- and post-operative measurements were available. The clinical pre-operative tumor size was 0.3 - 8.0 cm (mean 2.1 cm). The final size of

the Mohs surgical defect ranged in diameter from 1.0 - 11.5 cm (mean 3.8 cm) (Fig.2).

Fig.3 shows the locations of the BCCs as seen in our study population. The most frequent location was the nose (n=24; 33%), followed by the forehead/temple (n=15; 21%), the alar base/nasal-labial groove (n=13; 18%), the periocular and canthi region (n=7; 10%); the ear and pre/post auricular region (n=6; 8%), the cheek (n=4; 6%), the upper lip (n=2; 3%), and the trunk (n=1; 1%).

The following histologic patterns and their respective numbers and frequencies were observed: nodular type (n=27; 38%), infiltrative type (n=15; 21%), micro-nodular type (n=3; 4%), adenoid type (n=2; 3%), and basosquamous type (n=1; 1%). In the remaining 24 cases (33%) studied, circumscribed solid tumor masses of various sizes and shapes were observed in combination with deeply invading basaloid cells predominantly arranged as elongated strands, only a few layers thick with little or no palisading of the peripheral cells. Increase of stroma collagen or fibroblasts was not present (Fig.4). This 'mixed' tumor could be classified as nodular-infiltrating type BCC.¹² In all these 24 nodular-infiltrative type BCCs we compared the histopathological features from the center of the tumor with the horizontal and vertical tumor extensions. All cases demonstrated a shift from a circumscribed nodular growth pattern at the center of the tumor to a more infiltrative growth at the periphery, with extensions deep into the dermis, subcutaneous fat or muscle.

Fig.5 shows the distribution of the different histologic types of BCC, correlated to the different anatomic locations. Both the nose and nasolabial region show a preference for the nodular and nodular-infiltrative type, whereas the forehead displays a preference for the infiltrative type.

Next, the subclinical growth pattern of different histologic subtypes of BCC is presented. Among the nodular BCC types, 56% of the cases displayed horizontal subclinical growth, 26% vertical and 18% were characterized by both horizontal and vertical tumor extension. The infiltrative and mixed nodular/infiltrative types displayed the following subclinical patterns: horizontal (40%), vertical (40%), horizontal/vertical (20%), and horizontal (33%), vertical (25%), horizontal/vertical (42%) respectively.

Fig.6 shows the different histologic subtypes with their corresponding subclinical outgrowth pattern, related to different anatomic locations. The forehead in particular displays a horizontal growth pattern, whereas the nose is characterized by both horizontal and vertical extensions. The nasolabial area displays a striking pattern of vertical expansion.

Figure 1. Patients with recurrent extensive BCC according to sex and age.

Figure 2. Pre-operative tumor size and post operative wound size (n=56).

Figure 3. Anatomic distribution of 72 recurrent extensive BCCs.

Figure 4. Histological subtypes of 72 recurrent extensive BCCs.

Figure 5. Distribution of the different histologic types of BCC, correlated to the anatomic location (n=65).

Figure 6. Subclinical outgrowth patterns related to different anatomic locations (n=72).

Discussion

Both clinical as well as histologic features have been described to be of prognostic significance in predicting aggressive behaviour of a BCC.

The term 'aggressive' means the tendency for a BCC to recur after treatment and/or to invade deeply and widely, with resultant destruction or replacement of normal tissue.¹²

Besides recurrence of tumor, tumor size, tumor location and histological features also are correlated to aggressive tumor behaviour.

In this study we selected 72 cases of recurrent BCCs with extensive subclinical tumorgrowth, as demonstrated by at least three surgical Mohs stages before complete tumor removal was achieved.

First we will discuss the tumor size. Breuninger et al demonstrated that large tumors (> 2cm) display significantly wider subclinical growth and higher recurrence rates compared with smaller BCCs (< 1cm).¹¹ The mean preoperative tumor size in our study was 2.1 cm leading to a mean final surgical defect of 3.8 cm. Therefore the mean surgical margin needed for complete excision of these 72 recurrent BCCs proved to be 8.5 mm. Current surgical recommendations for primary BCCs call for margins of 2 to 5 mm in excision of nodular BCC and roughly 7 mm in infiltrative BCC, whereas for recurrent tumors an extra 2 to 3 mm should be added.^{13,14}

In this study we especially focused on tumor location and histological features as risk factors for aggressive tumor behaviour.

Occasional series in the literature have reported a statistically significant association between location and recurrence.^{6,8,15} Particular problem areas include the central 'H zone' of the face and certain scalp lesions. The explanation for higher recurrence rates in these areas is probably multifactorial, including 1) tendency of tumor to spread along embryonic fusion planes, facial planes, periosteum and perichondrium, resulting in subclinical extension of the tumor, and 2) inadequate excisions in cosmetically important areas.¹⁶ Indeed, ninety-nine percent of our recurrent tumors were located in the face, of which the nasal-perinasal area was the most common site (51%), followed by the forehead/temple region (21%).

With regard to the histological features, at least 26 histopathological subtypes have been defined.¹⁷ It was Thackray who first correlated the histologic classification of BCCs with prognosis.¹⁸ Infiltrative and micronodular growth patterns have been associated more with recurrence compared with the nodular types of BCC.¹⁹⁻²²

These differences in recurrence rates are believed to be due to better well-defined clinical borders of the nodular types, resulting in a higher likelihood of total excision. Solid and superficial BCCs do not differ in subclinical extension, whereas the more infiltrative types of BCC are characterized by larger, undetectable subclinical extensions that make them more difficult to excise completely.

However, of our 72 recurrent BCCs selected for their subclinical expansion, 27 cases (38%) demonstrated a purely nodular tumor growth, whereas only 15 cases (21%) were of the infiltrative type. These results indicate that nodular BCCs also can behave in an aggressive fashion with extensive subclinical growth. Besides a purely nodular or infiltrating growth pattern, in 24 cases (33%) a combination of both was observed. This type was mainly characterized by a central mass of large, rounded islands and poorly circumscribed margins with spiky nests lacking palisading. These tumors are classified as nodular-infiltrating BCCs.¹² We believe it is important to note these peripheral infiltrative elements because they appear to correlate with an increased tendency toward recurrence and biologically aggressive behaviour. Unfortunately, a diagnostic biopsy specimen initially taken of this type of tumor will show particularly a nodular growth pattern. Only the subsequent excision of the same BCC will demonstrate the peripheral infiltrating features, resulting in an increased risk of positive margins and a higher recurrence rate.

When we combine the results of the anatomic locations with the histological results, the nasolabial area (n=10) shows the most interesting outcome. Seventy-seven percent was of the nodular or nodular-infiltrative type, whereas the infiltrative type was not represented. 92% of the tumors was characterized by a vertical or a vertical/horizontal tumor expansion, whereas only 8% of the cases displayed a mainly horizontal growth pattern. The nose was almost equally represented by all different histological sub-types, with both horizontal and vertical tumor growth. Finally, the forehead displayed a predominance for horizontal subclinical extension of tumor.

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Surgical margins for excision of primary and recurrent basal cell carcinoma

M.E.J.M. Verhaegh, F.W.G. Gruitjens, G.A.M. Krekels,
A.H.M. Vermeulen, H.A.M. Neumann

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Summary

The literature is quite variable concerning recommendations as to the optimal surgical margins in the treatment of basal cell carcinoma (BCC).

Guidelines for surgical margins of a selected group of primary and recurrent BCCs were formulated on the basis of a retrospective study of 309 tumors treated by Mohs' micrographic surgery. To obtain the surgical margin, the maximal defect-diameter was subtracted by maximal tumor-diameter and divided by two.

In our group of primary BCCs selected for high-risk areas, large tumor size and/or aggressive histologic subtype, a minimum margin of 6 mm was required for complete removal of the tumor in more than 85% of the cases. The group of recurrent BCCs required margins of at least 10 mm to achieve a comparable tumor clearance.

Minimal margins of excision of 6 mm for primary and 10 mm for recurrent BCCs are proposed for our selected group of high risk tumors. Previous treatment (recurrent tumors) and large tumor diameter (larger than 2 cm) proved to be the two most important risk factors correlated with extensive subclinical tumor expansion. For these high risk tumors Mohs' micrographic surgery is the procedure of choice.

Introduction

BCC is the most common malignancy in humans, predominantly found on sun-exposed areas of the skin.¹ The incidence of BCC is rapidly increasing worldwide.^{2,3} In the Netherlands (15 million inhabitants), approximately 18.000 BCCs were diagnosed in 1994, with an estimated increase of 2.700 BCCs yearly.

BCCs are typically slow-growing tumors, often taking months to years to reach significant clinical proportions. Besides clinical proportions, BCCs also are characterized by varying subclinical tumor extensions. This is the reason that especially in BCCs the recommended margins of excision are quite variable, ranging from 2 mm to more than 1 cm.⁴⁻¹⁵

Most BCCs are relatively 'non-aggressive' tumors that are cured by various methods of treatment like conventional surgery, cryosurgery, curettage and electro-desiccation, and radiotherapy. Rowe et al described a recurrence rate for primary BCCs of 8.7% for all of the treatments above.¹⁶

A minority of tumors, however, display a more aggressive biological behaviour with a more extensive local tissue destruction and multiple recurrences, e.g. "high risk" BCCs. Factors that are correlated with aggressive tumor behaviour include tumor location, tumor size and histologic subtype.¹⁷

Mohs' micrographic surgery (MMS), a method that provides complete microscopic control of the surgical margin with a long term recurrence rate for primary BCCs of 1.0%, seems particularly useful for managing these aggressive-type BCCs.^{16,18}

MMS also is the ideal procedure for methodically mapping BCC growth patterns and so determinating the tumors' excision-margins. These characteristics will be used in this study of 309 BCCs, supported by a literature-overview. Proposals for surgical margins for primary as well as recurrent BCCs will be offered, in order to achieve tumor clearance rates of at least 85%.

Material and Methods

A retrospective study was performed on a total of 898 histologically confirmed BCCs treated with conventional surgery (n=589) or MMS (n=309) at the University Hospital Maastricht in the period between 1992 and 1996.

MMS is a standard treatment for BCCs in the United States of America. In Europe, where MMS is less widely available and accepted, only a selected group of BCCs is treated by this technique. Selection criteria applied in Maastricht are summarized in Table I.

The remaining group of BCCs (n=589), not selected for MMS, was treated by conventional surgery with a standard excision margin of 3 mm.

Table I. Selection criteria for Mohs' micrographic surgery applied in the University hospital Maastricht.

Recurrent tumor
Large tumors (> 2 cm) Particularly in cosmetically sensitive areas
Tumors with aggressive histopathology Subtype (infiltrative, micronodular, metatypical)
High-risk anatomic locations Nose, periorbital, ears Areas of embryonic folds (nasolabial fold)

The following parameters are available: age, sex, location, pre-operative tumor-size and post-operative defect size, vertical and horizontal infiltration type, histologic-subtype, previous therapies, reconstruction strategies, follow-up time and MMS-recurrence.

Anatomic tumor-locations were divided in forehead/temple, periocular and canthi, nose, paranasal/alar base/nasal-labial groove, cheek, ear and pre/post auricular and 'other' locations.

Pre-operative tumor sizes and post-operative defect sizes were measured during the MMS session. Maximal tumor-diameter and maximal defect-diameter were obtained by measuring the longest axe of tumor and defect. To obtain the tumor-margin, the maximal defect-diameter was subtracted by maximal tumor-diameter and divided by two.

Horizontal and vertical infiltration mode was adapted from the infiltration models of Breuninger et al.¹⁹

Histologic subtypes were divided in nodular BCC, infiltrating BCC, nodular/infiltrating BCC, micronodular BCC, superficial BCC, basosquamous BCC, adenoid BCC and category 'other' according to Weedon.²⁰

Previous therapies consisted of surgery, cryotherapy, radiotherapy, MMS, curettage and electrodesiccation or combinations of these.

Results

A series of 309 selected BCCs treated by MMS was reevaluated. The number of surgical Mohs stages was 1-8 (mean 2.4) and frozen slides 2-55 (mean 9.3). Of all patients 181 (59%) were male between the ages of 31 and 88 years (mean 64.2)

Table II. Histologic subtypes categorized by anatomic location.

	Forehead	Eye	Nose	Paranasal
Nodular	34% (n=20)	46% (n=20)	28% (n=29)	58% (n=19)
Infiltrative	33% (n=20)	20% (n=07)	46% (n=47)	11% (n=04)
Nodular/Infiltr.	09% (n=05)	13% (n=06)	15% (n=16)	18% (n=06)
Other	24% (n=14)	21% (n=10)	11% (n=11)	13% (n=04)
Total	100% (n=59)	100% (n=43)	100% (n=103)	100% (n=33)

and 128 (41%) were female between 31 and 94 years old (mean 67.5). The overall mean age was 65.5 years.

Of the 309 BCCs 138 (45%) were primary tumors. In the recurrent group (n=171); 127 (75%) tumors had been previously treated once, 21 (12%) twice, 9 (5%) three times and 14 (8%) tumors had been previously treated at least four times. These previous modalities of treatment included: surgery (n=90), cryotherapy (n=19), X-ray therapy (n=10), curettage-electrodesiccation (n=3), surgery and X-ray therapy (n=13), surgery and cryotherapy (n=11) and MMS (n=9).

Almost all BCCs were located in the face. The most frequent locations were the nose (n=103; 33%), the forehead/temple (n=59; 19%), the periocular and canthi region (n=43; 14%), the alar base/nasal-labial groove (n=33; 11%), the cheek (n=25; 8%), the ear and pre/post auricular region (n=21; 7%).

The following histologic patterns and their respective numbers and frequencies were observed: nodular type (n=111; 36%), infiltrative type (n=101; 33%), nodular/infiltrating type (n=49; 16%), micronodular type (n=11; 4%), superficial type (n=9; 3%), baso-squamous type (n=8; 2%), adenoid type (n=6; 2%) and category 'other' (n=14; 4%). The histologic subtypes are categorized by their anatomic location in Table II.

The pre-operative tumor size of both primary and recurrent BCCs was 2-100 mm (mean 15.9 mm). The final size of the Mohs surgical defect ranged in diameter from 5-150 mm (mean 27.5 mm). The mean tumor size and surgical defect for primary and recurrent BCCs respectively were 14.4 / 24.1 mm and 17.2 / 30.3 mm. The mean surgical margin for our selected group of BCCs treated by MMS was 5.8 mm. The mean surgical margin for primary and recurrent BCCs was 4.9 mm and 6.6 mm respectively.

The relation of tumor size and surgical margins was determined. Tumors were grouped according to maximum diameter: 0 to 9 mm, 10 to 19 mm, or 20 mm and larger. The mean surgical margins were 5.1, 5.2 and 7.4 mm respectively. The differences in surgical margins between primary and recurrent tumors according to tumor size also were determined. Primary BCCs (n=138); 3.8 mm (tumor size

Figure 1. Rate of tumor clearance for various margins of excision in 309 BCCs studied.

0-9 mm), 5.2 mm (tumor size 10-19 mm) and 5.9 mm (tumor size > 20 mm). Recurrent BCCs (n=171); 6.3, 5.2 and 8.1 mm respectively.

The effect of the three most common histologic subtypes on surgical margins also was studied. The mean surgical margin for nodular-type BCC (n=111) was 5.5 mm, for the infiltrative type (n=101) 4.9 mm and for the nodular/infiltrative type 7.1 mm.

The minimal lateral margins for clearance were determined in the 309 tumors studied. A 3 mm margin only cleared 27% of our BCCs, whereas our mean margin of 5.8 mm cleared 73% of the cases. The standard of 85% tumor clearance was achieved with 7.5 mm margins. The differences between primary and recurrent tumors also was studied (Fig. 1). A 6 mm margin cleared 85% of the BCCs in the group of primary tumors, whereas a margin of 10 mm was needed in the recurrent group. The rate of tumor clearance as function of tumor size and histologic tumor type of both primary and recurrent BCCs are illustrated in Figures 2-5.

Next, the subclinical growth pattern is presented. Among all BCC subtypes, 40% (n=125) of the cases displayed a predominantly horizontal subclinical growth, 23% (n=70) vertical and 14% (n=43) were characterized by both horizontal and vertical tumor extension. In 23% (n=71) of cases the subclinical growth pattern remained unknown because of a minimum of surgical Mohs stages.

The following reconstructions were performed: primary closure (n=58; 19%), advancement flap (n=49; 16%), transposition flap (n=41; 13%), rotation flap (n=33; 11%), subcutaneous pedicle flap (n=17; 6%), full-thickness graft (n=37; 12%), split-skin graft (n=42; 14%), secondary healing (n=4; 1%) and category other (n=25; 8%).

Until now eleven recurrences (3,5%) occurred, within a mean follow-up time of 28.7 months.

Figure 2. Rate of tumor clearance as function of tumor size in 138 primary BCCs.

Figure 3. Rate of tumor clearance as function of tumor size in 171 recurrent BCCs.

Figure 4. Rate of tumor clearance as function of tumor type in 122 primary BCCs.

Figure 5. Rate of tumor clearance as function of tumor type in 138 recurrent BCCs.

Discussion

The goals in the treatment of BCC are complete removal of the tumor and conserving normal tissue as much as possible. Since the probability of presence of tumor nests or strands beyond the clinical margins has been well documented,⁴⁻¹⁵ normal-appearing skin must also be removed or destroyed to achieve acceptable cure rates.

Until now only a few studies have been published in which the authors presented some recommendations regarding excision-margins for BCC.⁴⁻¹⁵ These recommendations varied from 2 mm to 10 mm (Table III). Unfortunately, in most of these studies important factors like recurrence, histology and location were not included and the correlation between the histologic and clinical extent of the tumor was not studied by 100% margin examination. In fact, MMS, with its inherent microscopic control of all tissue margins, seems the most appropriate method to determine subclinical extensions of BCCs.

The two most informative and well designed studies will be discussed. First, Wolf et al studied 117 cases of previously untreated, well-demarcated BCCs.¹³ Of the 117 cases, 11 lesions (9%) were greater than 2 cm and only a few cases displayed infiltrative features. Remarks concerning tumor location were not included in this study. Prior to excision, the normal-appearing skin surrounding the tumor was marked in 2 mm increments. This method was used because of the tendency of wound expansion following full-thickness tumor excision. All tumors were treated by MMS. For tumors with a diameter less than 2 cm, a margin of 3 mm would have eliminated the tumor completely in approximately 85% of the cases.

Breuninger et al, studied a total of 2016 BCCs.¹⁴ Of the 1757 primary BCCs, 177 (10%) were greater than 2 cm, 916 (52%) were exclusively nodular and 230 (13%) exclusively infiltrative. Histologic confirmation of the specimen margins took place with modified micrographic surgery and three-dimensional study in paraffin sections.²¹ For primary and recurrent tumors, margins of 4 mm and 6 mm respectively were required to achieve tumor clearance in 85% of the cases. Their results showed that the subclinical extension of BCCs is determined largely by the size of the tumor and the histologic type. Anatomic location and tumor age only indirectly affected subclinical extension.

At the University Hospital Maastricht, in the period 1992-1996, 898 BCCs were treated by surgical means. 309 of these 898 BCCs were selected for MMS (criteria given in Table I). The remaining 589 tumors, mostly small, previously untreated, well-defined, nodular type BCCs, were treated by surgical excision with a standard surgical margin of 3 mm. Post-operative histopathologic examination took place by vertical step sectioning (breadloaf method). In 104 of the 589 cases positive specimen margins were detected, resulting in a tumor clearance of 82%. One should keep in mind that this method of routinely vertical sectioning through rep-

Table III. Summary of advised surgical margins for BCC.

Author	Year	Type	Advice	Risk factor
Beirne	1959	169 BCC	5-10 mm	location (nose), infiltrative type
Macomber	1959	853 BCC	5 mm	—
Rintala	1971	204 BCC	5 mm	size of tumor
Griffith	1973	634 BCC	3-10 mm	size of tumor
Epstein	1973	131 BCC	2 mm	size of tumor, infiltrative type
Bart	1978	468 BCC	3-5 mm	location (periocular, scalp, nose, paranasal)
Koplin	1980	review	2-4 mm	size of tumor, location, recurrence
Albright	1982	review	2-10 mm	size of tumor
Breuninger	1984	742 BCC	2-5 mm	size of tumor
Wolf	1987	117 BCC	3-4 mm	size of tumor
Breuninger	1991	2016 BCC	3-7 mm	size of tumor, infiltrative type, recurrence
Hendrix Jr	1996	278 BCC	4-10 mm	infiltrative type, recurrence
Verhaegh	1998	309 BCC	6-10 mm	size of tumor, recurrence

representative areas of the specimen only permits the examination of less than 0.1% of the actual tissue margins.²²

Of the 309 BCCs treated by MMS, 171 were recurrent ones. Primary tumors were selected for MMS because of their size (larger than 2 cm), site (anatomic regions known for their increased risk of recurrence, or critical locations, such as the eyelid or lip, benefitting from the tissue-sparing aspects of the Mohs technique), and histologic subtype (infiltrative, micronodular, and mixed).

If this selected group of primary tumors would have been treated by the standard surgical margin of 3 mm, only 27% of the cases would have been excised radically. In this study we demonstrated that subclinical extensions of BCCs are mainly determined by previous treatment and the size of the tumor, especially tumors larger than 2 cm (Fig. 1-3).

Besides recurrent tumors and tumor size, the histological subtype also is described to be of significant importance concerning subclinical tumor extensions.²³

Recently, Hendrix et al showed, that the width of tissue required to remove subclinical extension of tumor was significantly greater with infiltrative BCC when compared with nodular BCC for primary tumors (5.6 mm vs 4.3 mm) and recurrent tumors (10.4 mm vs 5.7 mm).¹⁵ Our results only partly confirm their findings (Fig. 4,5). Especially the recurrent mixed type tumors (nodular and infiltrative characteristics in the same tumor) displayed extensive subclinical tumor growth.

Anatomic tumor location is not discussed here because of the fact that almost all our tumors were located in the so called central “H-zone” of the face, which is described to be correlated with a higher tumor aggressiveness.^{24,25} In our experience,

Table IV. Safety margins (mm) of primary and recurrent BCCs according Verhaegh et al and Breuninger et al that ensure tumor-free excisional margins in approximately 85% of the cases.

	Safety margin (mm)			
	Verhaegh et al (n=309)		Breuninger et al (n=2016)	
	Primary	Recurrent	Primary	Recurrent
Diameter (mm)				
< 10	4.5	8.0	3.5	—
10 - 20	7.0	8.5	4.5	—
> 20	10.0	15.0	7.0	—
Tumor type				
Nodular	5.0	8.0	4.0	—
Infiltrative	6.0	9.5	5.5	—
Nodular/Infiltr	5.0	12.5	—	—
Total	6.0	10.0	4.0	6.0

the most aggressive and destructive BCCs were localized in the nasal-labial groove and medial canthus areas. Worth mentioning, most of them were of the nodular type, and were characterized by extensive vertical tumor expansion.

In conclusion, the recommended safety margins of high risk BCCs (Table I) are summarized in Table IV. We have found by studying these 309 BCCs that a minimum margin of 6 mm for primary and 10 mm for recurrent BCCs was necessary to eradicate a tumor in 85% of the cases.

However, the methodology we used calculating the extent of the subclinical tumor invasion is correlated to some degree of variability. The subclinical extension was determined by dividing by half the difference between the maximal postoperative defect size and the maximal preoperative lesion size. This method does not compensate for wound expansion caused by gaping of the skin and/or orienting of the defect along the relaxed skin tension lines. Furthermore, our formula for the determination of the subclinical extension assumes that the extension of the tumor is symmetrical in all areas, although it is well known that subclinical extension in BCC is mostly asymmetric. In summary, presurgical marks with gentian violet at 2 mm increments along radial lines in all directions from the tumor borders, as described by Wolf et al,¹³ deserves the preference above our methodology.

To conclude, in our opinion MMS is the preferred technique for treatment of this group of high risk BCCs, because of the routine methodic accuracy in evaluating 100% of the surgical margins of the specimen, the subsequent high rate of oncologic cure, and the tissue-sparing quality of the procedure.

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General discussion and summary

In this final chapter an attempt will be made to summarize and discuss the results presented in this thesis in a somewhat broader context than was aimed in the discussion of the individual chapters. Three main themes will be addressed. First, our findings in chapters 2 and 3 are discussed in relation to the possible role of specific cell-mediated immunity as an important mechanism in limiting BCC tumor spread. Second, the role of programmed cell death regarding BCC tumor growth, studied by *bcl-2*, will be discussed by reviewing chapters 4 and 5. Finally, clinical and histological features of BCCs, and their consequences upon optimal therapeutic care are discussed in the context of chapters 6 and 7.

Basal cell carcinomas (BCCs) are frequently associated with a peritumoral mononuclear cell infiltrate. The infiltrate surrounding BCC tumor islands largely consists of T cells with a predominance of the CD4+/T-helper (Th) over the CD8+/T-cytotoxic (Tc) subset (Th/Tc ratio of 1.9 ± 0.8), similar to that found in typical delayed hypersensitivity reactions. Natural killer cells (NK) and B cells are seen in much lower numbers.

In the last decade, several investigators have attempted to elucidate the function of this cellular inflammatory infiltrate surrounding these skin cancers and its possible role in controlling tumor growth.

The relatively benign tumor-behaviour, areas of spontaneous regression and the therapeutic response to intralesional administration of cytokines, such as IL-2 and IFN- α , are in favour of a specific anti-tumor immune response in BCC. On the other hand, the observations that the stromal inflammatory reaction surrounding BCC is usually mild, T cells do not infiltrate BCC tumor lobules and the frequency of BCC in immunosuppressed patients is only slightly increased compared with squamous cell carcinoma, argue against a specific anti-tumor response.

In chapter 2, we investigated if BCC tumor cells and endothelial cells of the peritumoral vasculature express adhesion molecules.

A tumor specific cellular immune response is considered to be associated with adhesion molecule expression on endothelial cells for recruitment of T cells into the tissue, and on tumor cells for lymphocyte-target cell interaction.

Recent interest has focussed upon three cytokine-inducible leukocyte adhesion molecules, designated intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin.

ICAM-1, which is inducible by IFN- γ and TNF- α , is expressed by a wide variety of cells, including resting endothelial cells and binds virtually all circulating white cells. E-selectin and VCAM-1 expression correlates with endothelial cell activation and is both inducible by TNF- α and IL-1.

The expression and distribution of the adhesion molecules ICAM-1, VCAM-1 and E-selectin by microvascular endothelial cells and tumor cells, together with their leukocyte receptors LFA-1, VLA-4 and CLA respectively, were studied in 33 BCCs of different histological subtypes.

Endothelial ICAM-1 expression was only slightly increased compared to normal skin, whereas expression of endothelial VCAM-1 and E-selectin was low or absent in all BCCs examined. Peritumoral infiltrates contained mostly LFA-1 expressing lymphocytes, with minimal VLA-4 and CLA positivity.

In none of the cases studied adhesion molecule expression of BCC tumor cells was identified.

This observed failure to express adhesion molecules on tumor cells possibly enables malignant cells to escape immunosurveillance, by preventing binding of the T lymphocyte to the target cell.

The inability of BCC tumor cells to express adhesion molecules is not absolute. Taylor et al presented a study where they demonstrated induction of ICAM-1 expression on tumor cells after BCC tissue had been incubated *in vitro* with IFN- γ .¹

The absence of adhesion molecules on BCC tumor cells as well on the overlying epidermal keratinocytes as demonstrated in this study, together with the moderate ICAM-1 and minimal VCAM-1 or E-selectin expression on the surrounding peritumoral endothelial cells could be explained by insufficient *in vivo* cytokine levels. Two causal hypotheses of insufficient *in vivo* cytokine levels will be discussed.

First, activated T lymphocytes are an important source of cytokines, especially those T lymphocytes responding to tumor-antigen stimulation. Different stages of lymphocyte activation are characterized by the appearance of specific cell surface antigens, such as IL-2 receptor, transferrin receptor and HLA-DR expression. The presence of activated T cells surrounding BCC has been demonstrated; with up to 30% to 50% of cells expressing activation antigens on their surfaces.² Whether this only distinguishes them from naive T cells activated by general stimuli (for example epidermal ulceration, a characteristic feature of nodular type BCCs), or supports the concept of tumor-derived antigens initiating a cell-mediated host-specific response is as yet unclear.

Second, CD4+ T cells are recently subdivided into functional subsets (Th1 and Th2) based upon cytokine profiles. Th1 cells produce IL-2, IFN- γ and TNF- α , resulting in T cell proliferation and macrophage activation, characteristic of cell-

mediated immune responses. In contrast, Th2 cells produce IL-4, IL-5, and IL-10, cytokines that augment antibody responses. Recently, Yamamura et al demonstrated IL-4, IL-5, and IL-10 mRNAs in BCC lesions and concluded that a particular T cell population producing type-2 cytokines accumulates in BCC.³ Obviously, if Th1 cells are indeed in the minority in BCCs, a low level of IFN- γ may be the cause of absence of ICAM-1 expression in BCC. Thus, BCC progression may be assisted by the activation of Th2 cells involved in antibody formation, and suppression of a population of Th1 cells that normally contribute to tumor stasis or elimination.

In conclusion, besides identifying the presence of adhesion molecules on both vascular endothelial cells and BCC tumor cells, as demonstrated in this study, information about the composition of the infiltrate also is essential.

We would especially be interested in new techniques differentiating Th1 from Th2 cells, and also in new monoclonal antibodies differentiating immune effector cells specifically activated by tumor cells.

As mentioned above, the cellular inflammatory infiltrate surrounding BCCs, largely consists of CD4+/T-helper cells. This predominance of Th-cells over Tc-cells is an interesting observation, since Tc- and NK- cells are the effector cells in major histocompatibility complex (MHC)-restricted and non-MHC-restricted cellular cytotoxicity, respectively. Especially, these cytotoxic reactions are thought to be important in the elimination of tumor cells. The mechanism by which Th-cells mediate tumor regression remains unknown.

Our objective of the study presented in chapter 3 was to study the expression of granzyme B (grB) by cells present in immune infiltrates surrounding BCC, as an indicator of cytolytic activity against the tumor.

The presence of characteristic granules in the cytoplasm of cytotoxic T cells and natural killer cells correlates with activation and subsequent cytolytic potential of these cells *in vitro*. These cytoplasmic granules contain a pore-forming protein, perforin, and several homologous serine proteinases called granzymes. The action of granzymes results in the apoptotic death of the target cell.

We investigated the expression of grB in 10 cases of BCC, of which 8 were untreated and 2 had been treated once with intralesional interferon- $\alpha 2b$.

Despite the presence of significant numbers of CD3+ and CD8+ T cells, there was a striking scarcity of grB-positive cells in peritumoral BCC areas.

The small number of cells in the infiltrate that stained for grB appeared to be NK cells.

In the two cases of BCC treated with IFN- $\alpha 2b$, the number of grB-positive NK cells in perivascular areas appeared to be increased.

In contrast, the number of grB-positive Tc-cells in squamous cell carcinoma (n=5), melanoma (n=4), and seborrhoeic keratosis (n=3), was clearly increased compared to BCC.

To the best of our knowledge, our study represented the first report on expression of granzymes in immune infiltrates of human skin cancer. Recently, two new studies concerning perforin and granzymes were published. Deng et al studied perforin-expressing T cells in 40 BCCs.⁴ Only in ten specimens, very small numbers of perforin-positive cells were detected. Shimizu et al showed CD8+ infiltrating cells to contain grB in lichen planus.⁵ They suggested that these CD8+ T cells induce apoptosis of keratinocytes, resulting in the typical colloid bodies.

Besides the release of perforin and granzymes, cytotoxic T lymphocytes also are able to kill target cells by surface interaction between Fas ligand and Fas. This is a fascinating new field of research, especially since failure of BCC tumor cells to express the Fas antigen has been reported, thereby avoiding T cell killing by Fas ligand bearing cytotoxic T cells.⁶

In summary:

1) the resting state of peritumoral endothelial cells, 2) the absence of adhesion molecules on BCC tumor cells, and 3) the lack of grB expression of peritumoral Tc-cells, plead against a role of a specific anti-tumor response in BCC.

One could speculate that this is caused by an insufficient number of adequately activated T lymphocytes (with their corresponding cytokine profile), responding to a specific tumor-antigen stimulation.

BCCs are typically slow growing tumors, often taking months to years to reach significant proportions. Several explanations for this clinical behaviour have been proposed: 1) prolongation of the S-(DNA synthesis) phase of the cell cycle in tumor cells; 2) a small proliferation fraction in the tumor; 3) tumor regression in response to host immune factors; 4) prominent cell death by the phenomenon of apoptosis.

Option number three, tumor regression in response to host immune factors is already discussed above.

We will now focus on the critical balance between cell proliferation and cell death. In chapter 4, we investigated the expression of *bcl-2* in BCC and also in SCC of the skin. The proto-oncogene *bcl-2* encodes an inner mitochondrial membrane protein which shows a functional role of blocking programmed cell death, referred to as apoptosis. The protein is expressed in basal cells in normal human epithelium, but not in more superficial differentiated cells. It has been suggested that this pattern of expression assists survival of stem cells while preventing overaccumulation of differentiated cells.

Immunohistochemical localization using a monoclonal anti-*bcl-2* antibody revealed positive *bcl-2* expression of all BCCs (15 patients). SCC's failed to express

bcl-2 (5 patients). The positive *bcl-2* staining of BCC tumor cells supports the hypothesis that BCCs originate from the basal layer of the epidermis.

The prominent *bcl-2* expression of BCC tumor cells also suggests that inhibition of programmed cell death occurs, and would argue against the suggestion that prominent cell death in BCCs accounts for their typically slow growth rate.

It should be realized, however, that apoptosis is a complex process, in which several molecules besides *bcl-2* are involved. This issue is further discussed in the following section.

To elucidate the combined role of apoptosis and proliferation in BCC, we analyzed 6 cases of BCC in a pilot study (unpublished data). For the detection of apoptosis we used the terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end labeling (TUNEL) assay, which is based on the detection of the 3'-OH nicks produced by apoptotic DNA fragmentation. Proliferation was measured by staining with Ki-67, a monoclonal mouse anti-human antibody which reacts to a nuclear antigen present in all phases of the cell cycle except for the G₀ phase. In general, we observed very low percentages of TUNEL positive cells with a mean of 0.17% positive tumor cells. The mean proliferation index was 6.15%. Combining these two results, it appears that BCCs show a low proliferation index, in combination with a low apoptotic index, i.e., a low cell turnover rate. These results were supported by several other authors.^{7,8} Moreover, in our group of 6 BCCs, we observed Ki-67 reactivity especially restricted to the nuclei of three to five rows of peripheral cells localized at the base of the tumor nests. Unfortunately, this is a tumor area which often remains behind in the case of incomplete excision.

In history, the relationship between life and death already has challenged many philosophers, poets, and theologians. It is now a subject of special interest to cell and molecular biologists as well.

For example, the wild type *p53* gene is located at chromosome 17p and functions as a tumor suppressor gene by acting like a brake on tumor growth to allow optimal repair of damaged DNA. It is suggested that if this repair is not successful the *p53* gene induces apoptosis. The mutant version of *p53* gene is possibly enhancing the *bcl-2* action by further impairment of apoptosis.⁹ Interestingly, in BCC an aberrant expression of *p53* protein has been reported.¹⁰

David Norris stated in 1995: "I suspect that the issue of control of apoptosis induction in BCCs is more complex than is suggested by immunostaining for *bcl-2* alone."¹¹ Since then the following proteins were discovered. The protein *bax* is central to activation of the program of apoptosis.¹² Activation of *p53* induces the immediate up-regulation of *bax* and the rapid induction of apoptosis. *Bcl-2*, a homologue of *bax*, heterodimerizes *in vitro* with *bax* and decreases the induction of apoptosis by *bax* homodimeric complexes. Similar dimerization occurs between *bax* and *bcl-xL*, which also inhibits the formation of *bax* homodimers and the in-

duction of apoptosis. *Bad* is a newly described homologue of *bcl-2* that forms complexes with the inhibitory molecules *bcl-2* and *bcl-xL*.¹³ By binding to the inhibitory proteins *bcl-2* and *bcl-x*, *bad* promotes the formation of *bax* homodimers and promotes apoptosis. Finally, *bak* is the most recent proapoptotic member of the ever-expanding *bcl-2* gene family. *Bak* has been primarily found to enhance apoptotic cell death following an appropriate stimulus. Tomkova et al showed in a recent study negative *bak* expression in 20 BCCs studied.¹⁴

In conclusion, the high expression of the apoptosis-suppressing protein *bcl-2* together with the small percentage of apoptotic and actively proliferating tumor cells, may indicate that lack of apoptosis and not increased cell proliferation is an important factor in tumor expansion in BCC.

This is an interesting speculation since some authors suggest that inhibition of programmed cell death may lead to neoplastic growth at slower rates than growth induced by oncogenes that act by stimulating cell proliferation. This could possibly be a new explanation for the typical slow growth rate of BCC.¹⁵

However, the role of *bax*, *bcl-x*, *bad* and *bak* in BCC should be studied in the future before any firm conclusions can be drawn.

Besides the considerations concerning possible biological implications of *bcl-2* staining of BCC tumor cells, the immunohistochemical expression of *bcl-2* also can be used as a diagnostic marker. In chapter 5 we studied staining differences of *bcl-2*, transforming growth factor-beta (*TGF-β*), and *CD34* between BCC and trichoepithelioma (TE). TEs are benign tumors confined to the dermis and characterized by aggregates of neoplastic epithelial cells with limited follicular differentiation.

Both the clinical as well the histological distinction between TE and BCC can be a diagnostic dilemma, especially for the infundibulocystic type of BCC. This distinction is important because of the consequences for subsequent patient management. BCC displays a much more aggressive tumor behaviour than TE, and should be excised completely.

Recently, *bcl-2* and *CD34* expression already had been reported to be of diagnostic help in differentiating between TE and BCC. In addition to *bcl-2* and *CD34*, we selected *TGF-β* (Transforming Growth Factor-β) because of its pleiotropic effects on cellular differentiation and growth. Seventeen cases of TE / BCC were studied and the results of the hematoxylin-eosin stained histological sections compared with immunohistochemical analysis of consecutive sections stained with *TGF-β*, *bcl-2* and *CD34*. The combination of staining for *TGF-β* and *bcl-2* seemed to be most valuable in differentiating TE from BCC. In general, TE displayed *TGF-β* positivity combined with negative *bcl-2* staining whereas BCC demonstrated the opposite. The *CD34* staining pattern was considerably less consistent.

TGF-β is a multifunctional regulator of both cell growth and differentiation and has been shown to inhibit the growth of epithelial cells, stimulate mesenchymal cells and promote epithelial cell differentiation.

Glick et al studied *TGF-β1* and *TGF-β2* expression in murine skin and skin tumors.¹⁶ They showed that loss of expression of *TGF-β* from epidermal cells may be associated with increased proliferation and decreased apoptosis. If we extrapolate this to the human situation in BCC, loss of *TGF-β* expression could be a second mechanism responsible for extended cell survival besides expression of *bcl-2*. Moreover, the absence of *TGF-β* expression in BCC could also be correlated with the apparent failure of the tumor cells to differentiate.

One could speculate that the *bcl-2* positive/*TGF-β* negative cells in BCC are responsible for the biologically more aggressive tumor behaviour of these tumors, compared to the *bcl-2* negative/*TGF-β* positive cells in TE. So, tumors with a *bcl-2* positive/*TGF-β* negative staining pattern possibly deserve a more aggressive therapeutic approach.

In conclusion, the *TGF-β* staining pattern appears to be a helpful additional marker together with *bcl-2* in differentiating between TE and BCC. The demonstrated staining differences may relate to the distinct origin and biological behaviour of the two tumors and may therefore be of value in subsequent patient management.

BCC is the most common malignancy in man, predominantly found on sun-exposed areas. The incidence of BCC is rapidly increasing worldwide. In the Netherlands (15 million inhabitants), approximately 18.000 BCCs were diagnosed in 1994, with an expected increase of 2.700 BCCs yearly.

Most BCCs are small, slow growing, well defined lesions easily treated by various methods. On the other hand, there is also a group of aggressive BCCs characterized by extensive local invasion and disfigurement.

In chapter 6, possible risk factors, both clinically and histologically, were evaluated in a retrospective study of 72 recurrent extensive BCCs treated by Mohs' micrographic surgery (MMS). All selected cases required at least three surgical Mohs stages before complete tumor removal was achieved.

The mean preoperative tumor size in our study was 2.1 cm. All 72 tumors except one were located in the face, of which the nasal-perinasal area was the most common site (51%). In 33% of the cases the histologic pattern displayed a mixture of solid and infiltrative components in the same tumor, 38% demonstrated a purely nodular tumor growth, whereas 21% was of the infiltrative type.

Besides the documented aggressiveness of the morpheaform type BCC, we also demonstrated extensive tumor growth of both nodular and nodular-infiltrative

type BCC. Especially the perinasal area was characterized by extensive vertical tumor expansion of the nodular/nodular-infiltrative tumor type.

In chapter 7, guidelines for surgical margins for excision of primary and recurrent BCCs were formulated on the basis of a retrospective study of 309 tumors treated by MMS. In our group of primary BCCs selected for high-risk areas, large tumor size and/or infiltrative histologic subtype, a minimum margin of 6 mm was necessary for complete removal of the tumor in more than 85% of the cases. The group of recurrent BCCs required margins of at least 10mm to achieve a comparable tumor clearance.

Previous treatment (recurrent tumors) and increasing tumor diameter (larger than 2 cm), proved to be the two most important risk factors correlated with extensive subclinical tumor expansion.

First we will discuss reasonable cure rates, and how many BCCs require MMS. In this thesis surgical margins were calculated to achieve cure rates of 85%. Of course there will be a progressive improvement in cure rates as one takes wider and wider margins. If we took 15-to 30 mm margins with BCC, the cure rate would be nearly 100%, but we do not believe that the subsequent morbidity would justify such an aggressive approach. Because of the asymmetry of subclinical tumor extensions, whose precise location cannot be predicted, a comprehensive amount of normal tissue is always sacrificed when margins of this extent are taken. We believe that it might be reasonable to aim for a 85% to 90% cure rate of BCC. Surgeons getting a 99% cure rate for BCC are simply cutting out too much.

Overall, the percentage of nonmelanoma skin cancer treated by MMS amounts 30% in the United States. About 5% of Mohs surgeons believe that all BCCs require MMS, but most would agree that this is an excessive use of resources.¹⁷

In the Netherlands, MMS is quite controversial. Currently, only 1.6% of all BCCs are treated by MMS. Most of the criticism probably is based on the fact that people are not well informed. It has been said that "in its wonderful conceptual simplicity, MMS evokes a copying response from nearly every cutaneous surgeon who witnesses it".¹⁸ Arguments of opponents of the MMS procedure are the following; time consuming, expensive and surgical overutilization. However, MMS can be an effective use of physician time, depending on the infrastructure of your practice. With MMS and fresh-frozen tissue analysis it is possible to schedule four patients for MMS in the morning, and routinely all procedures, including reconstruction, are completed by afternoon. Miller et al, emphasizes that MMS is not only cost-effective but also usually less expensive than other types of therapy.¹⁹ Finally, a lot of dermatologists comment that Mohs surgeons use a bazooka to "cure" a flea. Yet, because some skin cancers have invisible extensions, huge surgical defects are sometimes necessary, even when the clinically apparent lesion may have been small. In other words, one of the reasons for a high cure rate with MMS, besides the meticulous histologic evaluation of margins, is the simple fact that

Mohs surgeons make bigger holes than the average dermatologist. Of course, some of these big holes are the consequence of previous inadequate treatment by others. In our opinion, MMS is the preferred technique for treatment of high risk BCCs as described in chapter 6 and 7, because of the routine methodic accuracy for evaluation 100% of the surgical margins of the specimen, the subsequent high rate of oncologic cure, and the tissue sparing quality of the procedure. Since the incidence of BCCs is rising, hospitals in the Netherlands should provide more MMS settings in order to manage the selected difficult cases.

Another point of discussion concerns the management of tumor-positive surgical margins. Consequently, even when performing the recommended wide margins as suggested in our study, still 15% of the cases will be excised incompletely. Bieley et al, reviewed 78 tumors treated by MMS because of margin involvement after primary excision of BCC to detect the presence or absence of residual tumor.²⁰ Residual tumor was found in 55% of the cases as defined by the need for two or more stages of MMS to achieve a tumor-free plane. Reported recurrence rates for incompletely excised BCCs vary between 33%-39%.²¹ Because of these figures, some authors advocate a conservative policy of close observation for clinical recurrence. One study even concluded that it was cost-effective to wait and see if a recurrence appears.²² Others believe in a selective retreating approach. They feel that features like anatomic location and histologic subtype should determine if further surgery is necessary or not. In this thesis, we want to state that disease-positive surgical margins should not be ignored. With a likelihood of tumor recurrence ranging from 33%-39%, wait and see is unacceptable, and therefore only in very limited situations should watchful waiting be encouraged. As Salasche stated, "leaving tumor behind expectantly is a mix of wishful thinking and Russian roulette".²³ Patients do not always return for follow up, which leads to delays in diagnosis, and when recurrences do develop, they are often deep and destructive.

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Samenvatting

Hoofdstuk 1 geeft een overzicht van de diverse aspecten van het basaalcelcarcinoom (BCC) en beschrijft het doel van het onderzoek dat in dit proefschrift wordt beschreven.

Het BCC is de meest gediagnostiseerde maligniteit in Nederland, met ongeveer 29.000 nieuwe gevallen per jaar. Het veranderde zon- en reisgedrag in de laatste decennia, de vergrijzing van de bevolking en de afname van de ozonlaag spelen mogelijk een rol bij de sterke toename van de BCC-incidentie. Het BCC komt vooral voor bij de blanke oudere mens op de aan zon blootgestelde huid.

Het BCC is een langzaam groeiende tumor, die slechts zelden metastaseert. Wanneer het BCC echter niet afdoende behandeld wordt, kan het lokaal een destructief en invasief karakter hebben en op die wijze veel schade aanrichten, zowel in functionele als in cosmetische zin.

Dit proefschrift is onder te verdelen in drie verschillende onderzoekslijnen waarbij met name aandacht wordt besteed aan zowel histopathologische als ook klinische aspecten betreffende de karakteristieke langzame groeiwijze van het BCC (1-6 mm groei per jaar).

Wij hebben onderzocht in hoeverre een specifieke cellulaire immuunrespons (hoofdstuk 2,3) en een omvangrijke celdood (hoofdstuk 4,5) mede verantwoordelijk zouden kunnen zijn voor deze langzame groeiwijze.

Tot slot zijn wij op zoek gegaan naar klinisch relevante risicofactoren van het BCC, en hebben wij een poging ondernomen tot het opstellen van therapeutische excisiemarges (hoofdstuk 6,7).

Het doel van het onderzoek dat in **hoofdstuk 2** beschreven wordt, was om de mogelijke rol van het ontstekingsinfiltraat gelocaliseerd rondom de BCC tumorvelden op waarde te schatten. Menig auteur houdt dit ontstekingsinfiltraat verantwoordelijk voor de langzame groeiwijze van het BCC, ondanks het feit dat de ontstekingscellen vaak op afstand van de tumorvelden gelocaliseerd zijn en niet de tumor binnendringen. Voor een effectieve interactie tussen ontstekingscellen (met name T cellen) en tumorcellen, is de expressie van verschillende bindingsmoleculen noodzakelijk. In dit hoofdstuk hebben wij de aanwezigheid van de bindingsmoleculen *ICAM-1*, *VCAM-1* en *E-selectin* op de omringende bloedvaten alsook op de tumorcellen onderzocht. Behoudens een lichte aankleuring van *ICAM-1* op de bloedvaten (endotheel), toonden met name de tumorcellen geen aankleuring.

Naast het onderzoeken van aan/afwezigheid van bindingsmoleculen, is het ook van belang geïnformeerd te zijn over functionele mogelijkheden van de ontstekingscellen. Zijn de ontstekingscellen ook in staat om de tumorcel te doden?

In hoofdstuk 3 hebben wij de aanwezigheid van *granzyme B* onderzocht bij T cellen. Alleen wanneer deze T cellen op adequate wijze door tumorcellen zijn geactiveerd, brengen deze T cellen *granzyme B* tot expressie, en zijn zij in staat de tumorcel te doden. Uit ons onderzoek kwam naar voren dat slechts enkele T cellen *granzyme B* positief waren.

De aangetoonde afwezigheid van bindingsmoleculen op BCC tumorcellen, samen met de minimale expressie van *granzyme B* op de omliggende T cellen, pleiten niet voor een belangrijke rol van het ontstekingsinfiltraat in het BCC ten aanzien van een specifieke tumorafweer.

Naast de cellulaire immuunrespons wordt ook celverlies in de vorm van geprogrammeerde celdood (apoptosis) als oorzaak voor de langzame groeiwijze van BCCs genoemd. Tumorgroei weerspiegelt een verstoring van het evenwicht tussen celaanmaak en celafbraak. In voorafgaande studies was de aandacht met name gericht op toename van celaanmaak (proliferatie). Momenteel gaat de interesse in de wereldliteratuur echter vooral uit naar celaccumulatie ten gevolge van verminderde celdood. In hoofdstuk 4 hebben wij de aanwezigheid van *bcl-2* onderzocht in BCC. *Bcl-2* is een oncogen dat cellen beschermt tegen geprogrammeerde celdood. Dit oncogen komt onder andere tot expressie in de basale cellen van de opperhuid. In de BCCs door ons onderzocht werd een duidelijke positiviteit voor *bcl-2* gevonden. Daarnaast bleek slechts 6.15 % van de tumorcellen proliferatief actief te zijn (Ki-67 kleuring). Deze resultaten wijzen er mogelijk op dat de groei van het BCC met name wordt veroorzaakt door verminderde celdood en niet als gevolg van celproliferatie. Dit is van belang omdat tumorgroei als gevolg van verminderde celdood geassocieerd zou zijn met een minder agressief tumorgedrag.

In hoofdstuk 5 blijkt *bcl-2* samen met *TGF- β* van diagnostische waarde te zijn in het onderscheid tussen BCC en trichoepithelioma. Beide huidtumoren zijn vaak op klinische gronden niet te differentiëren, echter het BCC is een kwaadaardige tumor terwijl het trichoepitheliom een goedaardige groeiwijze vertoont.

In hoofdstuk 6 worden mogelijke risicofactoren van het BCC beschreven. Uit onze patiëntenpopulatie die werd behandeld door middel van Mohs' micrografische chirurgie werden die tumoren geselecteerd waarbij sprake was van een recidief na eerdere behandeling, en waarbij tenminste drie Mohs-ronden noodzakelijk waren voordat het operatiegebied als tumorvrij werd afgegeven. De essentie van Mohs' micrografische chirurgie bestaat uit een goede grafische voorstelling van de tumorlokalisatie en het laagsgewijs excideren van de tumor. Van iedere Mohs sneede wordt het gehele resectievlak, door middel van horizontale vriescoupes, gecontroleerd. Het basisprincipe is maximale curatie bij minimale weefselbeschadiging. Belangrijkste risicofactoren waren: 1) grootte van de tumor, met name groter dan 2 cm, 2) lokalisatie, waarbij met name de tumoren gelokaliseerd ter hoogte van de nasolabiaalplooi gekenmerkt werden door uitgebreide verticale diepte-groei, en 3)

histologische groeiwijze, waarbij naast de sprieterige groeiwijze, ook bij het nodulaire type uitgebreide subklinische tumorgroei werd waargenomen.

In hoofdstuk 7 worden richtlijnen geformuleerd betreffende excisie-marges voor primaire- en recidief BCCs, op basis van een retrospectieve studie van 309 BCCs door ons behandeld door middel van Mohs' micrografische chirurgie. Bij de groep primaire BCCs, door ons geselecteerd voor Mohs' micrografische chirurgie in verband met grootte van de tumor (> 2 cm), sprieterige histologische groeiwijze en/of lokalisatie in het centrum van het gelaat, werd een minimale excisie-marge van 6 mm berekend, om een radicaliteitspercentage van 85 % te bereiken. Bij de groep van recidief tumoren betrof dit 10 mm.

Tot slot kan geconcludeerd worden dat bij deze geselecteerde groep BCCs met deze subklinische uitbreiding, behandeling door middel van Mohs' micrografische chirurgie de voorkeur verdient.

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Wat mij nu rest is de aan *Jesje* toegezegde grotere rol in het huishouden (in de breedste zin van het woord...), of misschien toch maar golfen?

Curriculum Vitae

Marc Verhaegh werd geboren op 19 april 1962 te Groningen. Zijn Atheneum- β opleiding volgde hij aan het Jeanne d'Arc lyceum te Maastricht, waar in 1982 het diploma werd behaald.

In 1983 begon hij aan de Rijksuniversiteit Limburg zijn studie Geneeskunde. Het doctoraal examen werd afgelegd in 1987.

Tijdens zijn studie Geneeskunde was hij studentlid van de onderwijscommissie, bestuurslid van de Nieuwe Studenten Vertegenwoordiging Rijksuniversiteit Limburg, studentlid van de Stichting Wetenschappelijke Opleiding Medische Studenten en lid van de commissie de Blauwe Spiraal.

Verder vervulde hij gedurende twee jaar een student-assistentchap bij de vakgroep Farmacologie (hoofd: Prof.dr.H.A.J.Struyker Boudier) en verrichtte hij o.a. onderzoek naar het vaso-actieve effect van Platelet Activating Factor op de thoracale aorta van normotensieve ratten.

In augustus 1987 onderzocht hij het werkingsmechanisme van Pinacidil, een nieuw ontwikkelde vasodilatator met een klinische antihypertensieve werking, aan de vakgroep Biofysica te Aarhus, Denemarken.

Vanaf september 1989 tot februari 1990 was hij werkzaam in het St. James Mission Hospital te Mantsonyane, Lesotho, waar hij o.a. onderzoek verrichtte naar de drinkwaterkwaliteit.

In maart 1990 werd het arts-examen afgelegd.

Vanaf april 1990 is hij werkzaam op de afdeling Dermatologie van het Academisch Ziekenhuis Maastricht. Eerst als AGNIO en vanaf november 1993 als AGIO (opleider: Prof.dr.H.A.M.Neumann).

Eind 1992 doorliep hij gedurende twee maanden een "Mohs"-stage bij Dr. Antonio Picoto te Lissabon, Portugal.

Na zijn registratie als dermato-veneroloog op 1 mei 1997 is hij als staflid binnen dezelfde vakgroep werkzaam, met als aandachtsgebieden de dermato-oncologie en dermato-chirurgie.

Met ingang van juni 1998 is hij aangesteld als chef de policlinique.

Hij is getrouwd met Jesje Schalm, oefentherapeut-Mensendieck, en vader van Emily en Marije.

List of abbreviations

AG	: aggressive growth
BCC	: basal cell carcinoma
BCL-2	: B-cell leukemia/lymphoma-2
CLA	: cutaneous lymphocyte-associated antigen
grB	: granzyme B
HLA	: human lymphocyte antigen
ICAM	: intercellular adhesion molecule
IFN	: interferon
IL	: interleukin
LFA	: lymphocyte function-associated antigen
MMS	: Mohs' micrographic surgery
NBCCS	: nevoid basal cell carcinoma syndrome
NK	: natural killer
SCC	: squamous cell carcinoma
SK	: seborrhoeic keratosis
TE	: trichoepithelioma
TGF	: transforming growth factor
TNF	: tumor necrosis factor
TUNEL	: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling
UV	: ultraviolet
VCAM	: vascular cell adhesion molecule
VLA	: very late antigen