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Solid Basal Cell Epithelioma (BCE) Possibly Originates from the Outer Root Sheath of the Hair Follicle

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The presence and distribution of several cytokeratins (CKs) in 20 solid basal cell epitheliomas (BCEs) were investigated and compared with the pattern of CKs in normal epidermis, perilesional skin and in the outer root sheath (ORS) of the human hair follicle. Tissue samples were stained with monoclonal antibodies (MoAbs) against human CKs, using the APAAP technique. Additionally, CK profiles were assessed by gel electrophoresis and immunoblot technique. Cells of BCE and ORS were positively stained with the MoAb KL1, whereas the basal layer of normal epidermis remained negative. Six out of 20 BCEs were partially stained with the MoAb RPN1165, which also stained the lower ORS, but not the epidermal basal layer. Using SDS-PAGE and immunoblot, the CK profiles of BCE and ORS were almost identical, showing the presence of CKs 5, 6, 14, 16 and 17; the CK pattern of normal epidermis, however, showed the presence of CKs 1, 5, 10 and 14. Perilesional skin (<5 mm) showed keratin changes similar to the BCE pattern: the basal layer was stained with the MoAb KL1 and the suprabasal layer was negative to MoAb CK1, in contrast to normal epidermis. Keratin analysis revealed a CK pattern of perilesional skin different from that of normal epidermis (CKs 1, 5, 6, 10, 14, 16 and 17). Our immunohistochemical and biochemical investigations underline the possible role of the lower ORS as a cellular pool for the generation of BCE. ***Key words: Cytokeratins; SDS-PAGE; Immunohistochemistry.***

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Basal cell epithelioma (BCE) is the most common skin neoplasm, occurring almost exclusively in hair-bearing skin (1). Its incidence appears continuously increasing world-wide and UV light is suspected to be one of the promoting factors. Despite numerous previous investigations, consensus concerning the origin and differentiation of this semimalignant tumor is still lacking (2). The term BCE was chosen because of the morphological similarities of the tumor cells to basal keratinocytes: BCE was thus regarded as a basal cell layer-derived neoplasm. However, based on recent studies on intermediate filaments (IF) (3, 4), a close relationship between BCE and the hair follicle may be suggested.

The epithelial cytokeratins (CK), one subclass of IF, consist of 20 distinct proteins with a molecular weight ranging from 40 to 68 kilodaltons (kD) (5). They are regarded as major cytoskeletal components of keratinocytes, and biochemical studies on their exact function and distribution in human tissue have gained much interest in recent years. In particular, the CK pattern of epithelial cells has been used to determine cell origin and to evaluate cell differentiation in vivo and in vitro (6).

In this study we investigated the presence and distribution of CKs in BCEs, normal epidermis and in the outer root sheath (ORS) of the human hair follicle, using immunohistochemical staining, 1-D and 2-D gel electrophoresis and, in some cases, immunoblots. Perilesional skin surrounding the BCEs was included in our investigations in view of the high number of tumor relapses after surgical excision (7).

MATERIAL AND METHODS

Twenty solid BCEs, including 5 mm of normal appearing perilesional skin, of varying body sites (12× face, 4× scalp, 3× trunk, 1× lower leg) and 10 biopsies of healthy scalp skin were obtained by surgical excision and were divided for immunohistochemical and biochemical studies. Anagen hair follicles from 10 volunteers were obtained by plucking, using the standard trichogram technique.

Immunohistochemical staining

The tissue samples were frozen immediately in liquid nitrogen (−196°C), were cut into sections of 5–7 μm thickness and placed on albumine-coated glass slides. After drying at room temperature overnight, slides were fixed in aceton for 10 min and were then processed for immunohistochemical staining, using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique according to standard procedures introduced by Cordell et al. (8). The MoAbs against various human CKs used in this study are listed in Table I. The dilutions of each MoAb were determined according to the manufacturer's recommendation and modified after our experiments. Slides were counterstained with hemalum and were mounted with glycerol gelatine for light microscopic evaluation.

Cytokeratin extraction by gel electrophoresis

Tumor nests of solid BCEs were carefully isolated from the surrounding tissue with fine needles, using a stereo-dissection microscope (Zeiss, Germany). Tissue samples were cut into small pieces and washed several times in TRIS-buffered saline (TBS) to remove blood and other contaminants. Perilesional skin surrounding the tumor and biopsies of normal skin were treated with heat (60°C) for 10 min to separate the epidermis from the dermis.

Scalp hairs of healthy volunteers were plucked, using the trichogram technique and anagen follicles were carefully selected under the light microscope. They were immersed in TBS and a single cell suspension of ORS cells was obtained enzymatically using trypsin (0.25% in TBS, 15 min incubation at 37°C).

All samples obtained were homogenized and were sonificated (3 × 30 s) subsequently in a 3-buffer series: a) 20 mM TRIS/HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride; b) 600 mM KCl, 5 mM ethylenedinitrilotetraacetic-acid (EDTA), 5 mM ethylenebis (oxyethylenenitrilo) tetraacetic-acid (EGTA); and c) 50 mM TRIS/HCl, pH 7.4, 1% Triton X-100.

Table I. *List of monoclonal antibodies (MoAbs) against human cytokeratins (CKs) used in this study: their commercial sources, specificity and working dilutions*

MoAbs	Clone	Ig Class	Source	Specificity (CKs)	Dilution
CK1	34βB4	Ig G1	Enzo	1	1:100
CK8.60	K8.60	Ig G1	Bio Makor	10, 11	1:200
KL1	KL-1	Ig G1	Immunotech	10, 11	1:400
PKK2	PKK2	Ig G1	Labsystems	7, 16, 17, 19	1:200
CK8.12	K8.12	Ig G1	Bio Makor	13, 16	1:100
CK8.13	K8.13	Ig G2a	Bio Makor	1, 5, 6, 7, 8, 10, 11, 18	1:100
CK14	CKB1	Ig M	SIGMA	14	1:100
RPN1160	LE65	Ig G2a	Amersham	18	1:200
RPN1162	a3(CK7)	Ig G1	Amersham	7	1:200
RPN1165	LP2K	Ig G2b	Amersham	19	1:200
RPN1166	LE41	Ig G1	Amersham	8	1:200
Anti-cytokeratin	AE1 and AE3	Ig G1	Boehringer Mannheim	10/11, 14/15 1/2, 5	1:100

Protease inhibitors (5 μg/ml pepstatin and 5 μg/ml antipain) were added to all buffers. Each extraction step included ultrasonic disintegration and centrifugation at 20000× g for 12 min. The resulting pellets, highly enriched in cytoskeletal proteins, were solubilized in the respective samples of buffer consisting of 0.5 M TRIS/HCl (pH 6.8), 2.1% SDS, 10% mercaptoethanol, 10% glycerol and bromphenolblue and were heated for 10 min at 96°C (9).

One-dimensional sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (10) and two-dimensional analysis by non-equilibrium pH gradient electrophoresis (NEPHGE, 2 DE) according to O'Farrel et al. (11) were performed. Gels were stained in 0.2% Serva Blue R.

Immunoblot analysis

All keratins detected by Coomassie staining were characterized with the immunoblot technique using antikeratin monoclonal antibodies (MoAbs). Unstained gels were mounted on nitrocellulose paper, to

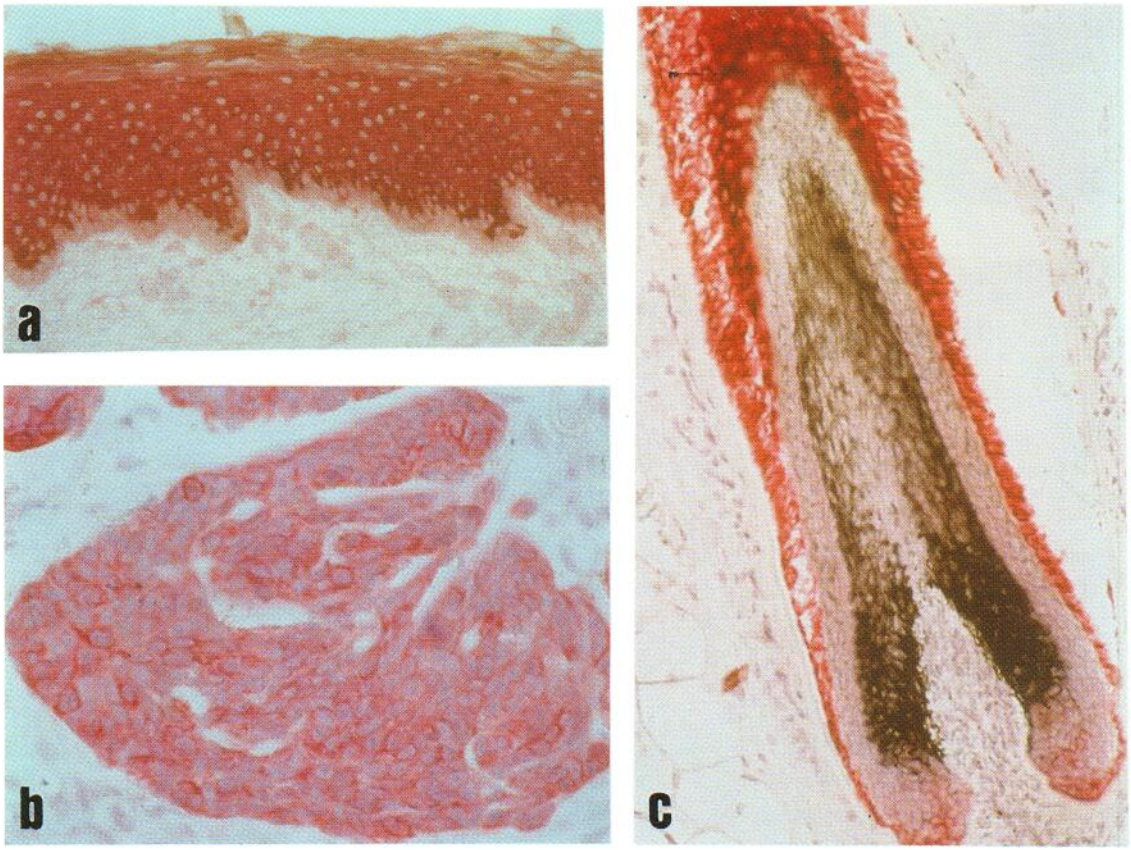

Fig. 1. Cryostate sections stained with MoAb KL1 (APAAP, 1:400). *a*: The basal layer of normal epidermis is not stained (×160). *b*: BCE cells are homogeneously stained (×160). *c*: ORS cells are stained only below the opening of the sebaceous duct (×80).

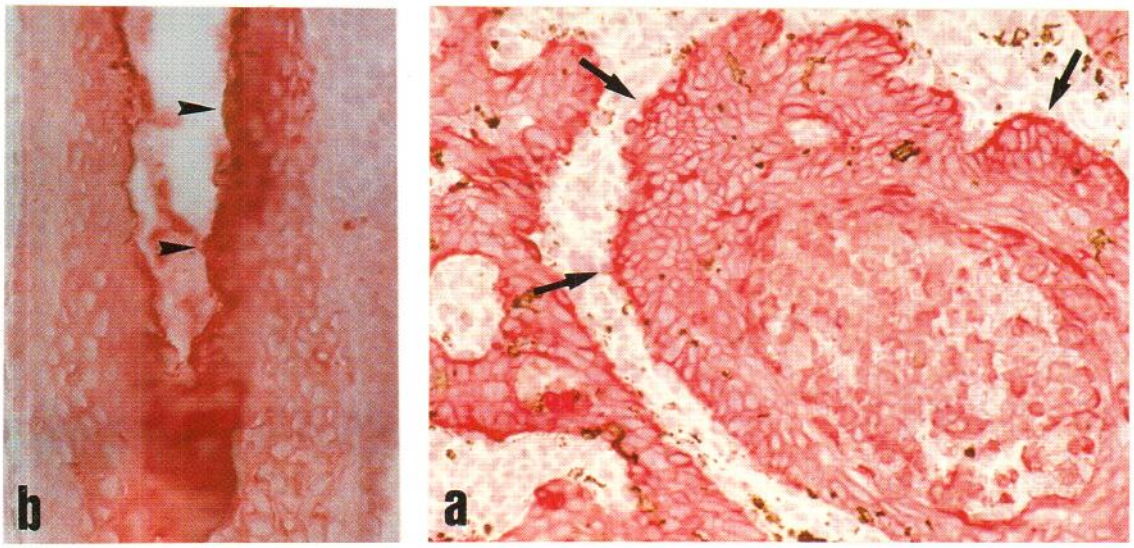

Fig. 2. Cryostate sections stained with MoAb CK8.12 (APAAP, 1:100). *a*: The outermost palisade-like cells of BCE (*arrows*) show marked staining (×160). *b*: ORS of the human hair follicle. A strong reaction is especially observed in the area of trichilemmal keratinization (*arrows*) (×160).

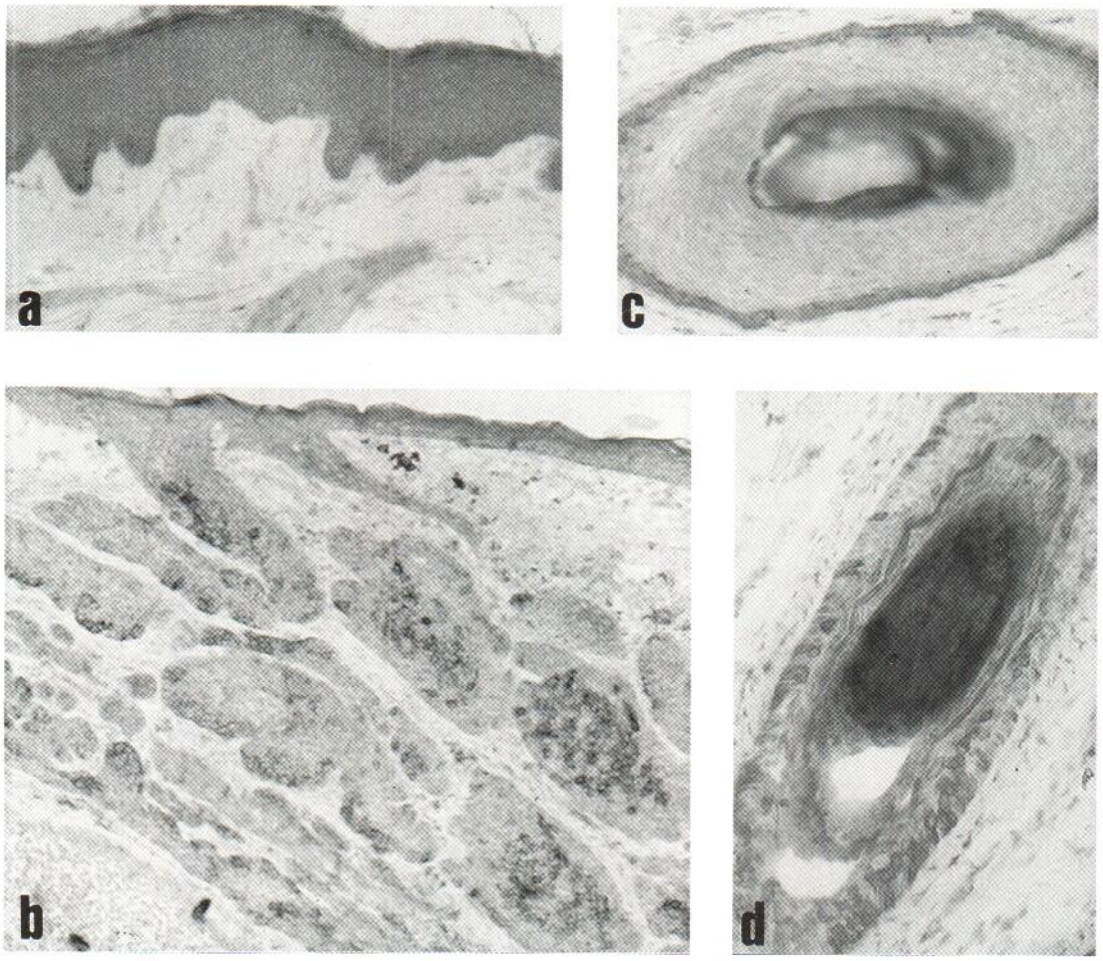

Fig. 3. Cryostate sections stained with MoAb RPN1165 (APAAP, 1:200). *a*: Normal epidermis shows no staining (×125). *b*: BCE cells are partially stained (×40). *c*: The outermost cells of the upper 2/3 of the ORS below the opening of the sebaceous duct are stained (×100). *d*: Cells located more centrally in the lowest 1/3 of the ORS show dotted staining (×80).

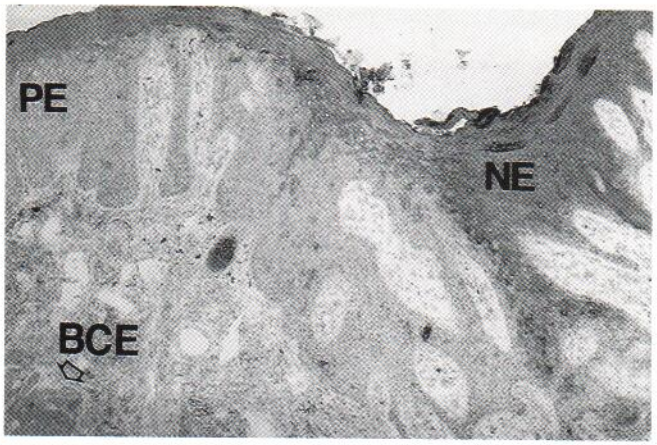

Fig. 4. Cryostate sections stained with MoAb CK1 (APAAP, 1:100). BCE cells are completely negative (*arrow*). The suprabasal layer of normal epidermis (*NE*) is positively stained, whereby the staining intensity fades in perilesional epidermis (*PE*) towards the tumor nests (×40).

which the proteins were transferred electrophoretically (12). After blocking unspecific protein-binding sites with 0.14 M TBS (10 mM TRIS/HCl pH 7.4 and 140 mM NaCl), supplemented with 0.05% Tween 20 and 15% horse serum, the membranes were incubated overnight with antikeratin MoAbs and with horseradish peroxydase-conjugated goat anti-rabbit immunoglobulin fractions (Bio-Rad, Munich, Germany). The peroxidase reactions were visualized using 4-chloro-1-naphthol and hydrogen peroxide.

RESULTS

Immunohistochemical findings

All 20 BCEs examined in this study were stained with the MoAbs KL1, PKK2, CK8.12, CK14 and CK8.13. In contrast, the basal layer of the epidermis was negative to MoAb KL1 (Fig. 1a), whereas BCE cells (Fig. 1b) and ORS cells of the hair follicle below the opening of the sebaceous duct (Fig. 1c) showed strong positive staining with this MoAb. A pronounced staining of the outermost (palisade-like) cell layer of BCE nests was observed with the MoAb CK8.12 (Fig. 2a), which only showed minor and variable staining of the basal layer of normal epidermis. Staining of the ORS of the hair follicles with the MoAb CK8.12 was stronger in the area of trichilemmal keratinization than elsewhere (Fig. 2b). As expected, the CK pattern of the ORS showed marked differences between its lower part below the opening of the sebaceous duct and the upper part of the hair follicle. The MoAbs KL1, PKK2, CK8.12, CK14 and CK8.13 showed positive staining in the lower parts of the ORS, similar to solid BCEs, the upper parts of the ORS showing a CK pattern identical with that of normal epidermis.

BCE cells were negative with MoAbs CK1 and CK8.60, which are markers for keratinizing epithelia and which thus stained the suprabasal layers of normal epidermis. These two MoAbs also reacted with cells located in the upper part of the ORS, as they did with the suprabasal layers of normal epidermis, but not with the cells located in the lower part of the ORS below the isthmus.

The MoAb RPN1165 was completely negative in normal epidermis (Fig. 3a), but 6 out of 20 BCEs were partially reac-

Table III. *Overview of the cytokeratin patterns of solid BCE perilesional skin, normal skin and the ORS of the human hair follicle, as detected by SDS-PAGE and immunoblot*

Keratin (CKs)	Normal epidermis	Perilesional skin	BCE	Lower ORS
1	+	+	–	–
5	+	+	+	+
6	–	+	+	+
8	–	–	(+)	–
10	+	+	–	–
14	+	+	+	+
15	–	–	(+)	–
16	–	+	+	+
17	–	(+)	+	+
19	–	–	–	–

Table II. *Overview of immunohistochemical findings in cryostate sections of BCE, perilesional skin, normal epidermis and the ORS of the hair follicle with MoAbs against human cytokeratins using the APAAP technique*

MoAbs	Normal epidermis		Perilesional skin		BCE	Lower ORS
	Basal layer	Suprabasal layer	Basal layer	Suprabasal layer		
CK1	–	+++	–	++ → –	–	–
CK8.60	–	+++	–	++ → –	–	–
KL1	–	+++	++	+++	+++	+++
PKK2	+++	–	+++	++	+++	+++
CK8.12	(+++)	–	+++	++	+++	+++
CK14	+++	–	+++	++	+++	+++
CK8.13	+++	+++	+++	+++	+++	+++
RPN1160	–	–	–	–	–	–
RPN1162	–	–	–	–	–	–
RPN1165	–	–	–	+	+	upper 2/3; – lower 1/3; +++
RPN1166	–	–	–	–	–	–

–: A positive reaction was observed in 0/20; +: 1–6/20; ++: 7–14/20; +++: 15–20/20; ( ): The staining intensity was weak.

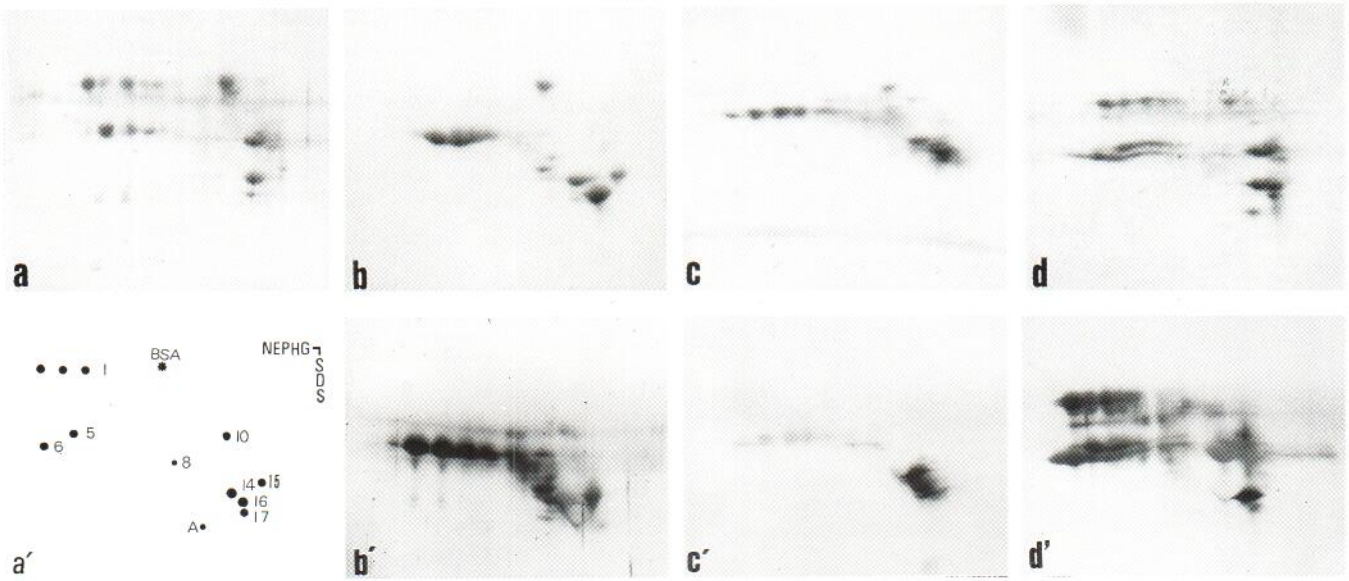

Fig. 5. Separation of CKs from BCE, perilesional epidermis and the ORS of the human hair follicle using SDS-PAGE (*a–d*) and immunoblot (incubation with MoAbs CK8.12, CK19, CK14, CK7, CK8, PKK2, CK8.60, CK8.13 and AE1/AE3) (*b'*, *c'*, *d'*). *a*: Normal epidermis expresses CKs 1,5, 10 and 14. *a'*: Diagram of keratins expressed in the study. *b, b'*: BCE cells express CKs 5, 6, 8, 14, 15, 16 and 17. *c, c'*: ORS cells express CKs 5, 6, 14, 16 and 17. *d, d'*: Perilesional epidermis surrounding BCE (<5 mm) expresses CKs 1, 5, 6, 10, 14, 16 and 17. *A*: Actin. *BSA*: Bovine serum albumin.

tive with this MoAb (Fig. 3b), which specifically stains CK19. RPN1165 also reacted with the outermost cells of the upper 2/3 of the lower ORS and with more centrally located cells in the lowest 1/3 of the ORS (Fig. 3c, d). The suprabasal layers of normal-appearing skin surrounding the BCE showed positive staining with the MoAbs KL1, PKK2, CK8.12, CK14 and CK8.13, corresponding to the CK pattern of BCE. However, positive staining with the MoAbs CK1 and CK8.60 was detected additionally, corresponding to the staining pattern of normal epidermis. In perilesional skin, the reaction with these two MoAbs clearly faded near the BCE tumor tissue (Fig. 4). No reaction was observed in BCEs and the lower ORS with the MoAbs RPN1160, RPN1162 and RPN1166. Our immunohistochemical findings are summarized in Table II.

Keratin profiles

Separation of CKs by SDS-PAGE (see Table III) displayed a cytokeratin profile of normal epidermis, consisting of CK1 (68000 kD), CK5 (58000 kD), CK10 (56000 kD) and CK14 (50000 kD) (Fig. 5a). In contrast, CK5, CK6 (56000 kD), CK16 (48000 kD) and CK17 (46000 kD) were extracted from BCE cells, while the large keratins CK1 and CK10 were absent. Trace amounts of CK8 (52500 kD) were detected in 6 out of 20 BCEs and CK15 was found in 2 of 20 BCEs (Fig. 5b). Using the immunoblot technique, positive reactions were observed with MoAbs CK8.12, PKK2, CK8 and AE1/AE3, detecting CKs 5, 6, 8, 15, 16 and 17. CK14 was weakly visualized by MoAb CK14. No reactions were detected with the MoAb RPN1165, which reacts with CK19 (40000 kD) or with MoAbs CK8.60 and CK1, reacting with the large molecular keratins (Fig. 5b').

SDS analysis of ORS cells from plucked human anagen hairs also revealed the absence of CKs 1 and 10, while CKs 5, 6, 14, 16 and 17 were expressed (Fig. 5c). CKs 5, 6, 14, 16 and 17 were confirmed by immunoblot analysis using MoAbs CK8.12, CK8.13, CK14 and PKK2 (Fig. 5c'). No additional reactions were observed with the MoAb against CK19 or with MoAb AE1/AE3. Perilesional epidermis was characterized by a cytokeratin pattern consisting of CKs 1, 5, 6, 10, 14, 16 and 17 (Fig. 5d, d'), which were ascertained by immunoblot using MoAb CK8.12, CK14, PKK2 and CK8.60. No reactions were additionally found with MoAbs CK7, CK8, CK8.13 or AE1/AE3.

DISCUSSION

The cellular origin of BCE is still a matter of controversy in spite of its early recognition as a "basal cell cancer", as suggested by Krompecher (13). BCEs are largely believed to be nevoid tumors which originate from incompletely differentiated, immature cells from the epithelial germ (1); also, a multiple-point origin from the epidermal basal layer and from skin appendages has been assumed by others (14). Recently, additional information on the biological aspects of tumor-differentiation has been obtained, and similarities between the cytoskeleton of BCE cells and cells of the pilosebaceous unit have been observed (3).

We (15) and Heid et al. (16) have already reported on different keratin patterns of the ORS of the human hair follicle regarding its location above and below the opening of the sebaceous duct. In this study, we could show the differences in keratin pattern between BCE and normal epidermis and the similarities between BCE and ORS located below the opening of the sebaceous duct, using immunohistochemistry and gel electrophoresis.

BCE cells and ORS cells were stained with the MoAb KL1, whereas the basal layer of normal epidermis remained negative. The positive reaction of BCE and of ORS with this

MoAb could be due to the presence of CKs 6 and/or 17, as confirmed by us with 2-D gel electrophoresis and immunoblot. The MoAb CK8.12 reacts with CK13 and CK16 (see Table II) and, in addition, a cross reaction with CK15 is known as a minor staining in the basal layer of normal epidermis (17). The expression of CK13 is apparently limited to the suprabasal layers of non-keratinizing squamous epithelia (5). SDS-PAGE showed the presence of CK16 and the absence of CK13 in BCE, in lower ORS and in perilesional epidermis. Thus, we concluded that the strong reaction of BCE cells with MoAb CK8.12 observed in our study is due to the presence of CK16. CK16 (type I keratin) is associated with CK6 (type II keratin) in a "cytokeratin-pair". Abnormal expression of this cytokeratin-pair has been seen to occur under several conditions, and the conclusion was drawn that it is a marker for hyperproliferation (e.g. wound-healing, psoriasis, in vitro cell culture) and/or for malignant transformation (18–21). In our investigation, the outermost palisade-like cells of BCE showed strong and pronounced staining with MoAb CK8.12 (Fig. 2a). This may indicate high proliferative activity of these cells rather than of cells located in the center of the tumor, as suggested by Grimwood et al. (22).

CK1 and CK10 were absent in both BCE cells and lower ORS cells immunohistochemically and biochemically. These cytokeratins are regarded as markers for keratinizing epithelia (6), since they occur in the suprabasal layers of normal epidermis and the upper ORS.

Both the increase of CKs 6 and 16 and the decrease of CKs 1 and 10 were also seen in normal-appearing skin surrounding the tumor. Winter et al. proposed that the reduction of large keratins together with an increase of low molecular keratins could be regarded as a biochemical tool for the discrimination of premalignant keratinocytic lesions in man (23). It is well known that increased synthesis of CK6 and CK16, and in some cases also of CK17, occurs after skin injury or otherwise induced hyperproliferation and in malignant transformation of keratinocytes (20, 21, 24). It may therefore be that these hyperproliferation-associated keratins in perilesional skin (<5 mm) are related to the observed high recurrence rates of BCEs seen after surgical treatment (25).

In some BCEs, CK8 was detected in a small amount using two-dimensional gel electrophoresis, which was supported by immunoblotting. This keratin is normally expressed in simple epithelia and is also observed in tumors derived from non-keratinized tissues and in cultured cells (5). Lavrijsen et al. reported the positive reaction of BCE cells with MoAb anti-cytokeratin M-20, which detects CK8 (26). On the other hand, MoAb RPN1166 did not stain any of the 20 BCEs. Habets et al. (27) also reported the lack of RPN1166 reaction with BCE cells in some cases. This discrepancy is possibly due to the reaction of this MoAb to a different epitope of CK8, which may not be present in BCEs. In the present study, CK8 was not detected electrophoretically in ORS cells. However, Heid et al. reported that CK8 is synthesized in the ORS of the lower hair bulb but that its amount appears too small to be detected by normal gel electrophoretic techniques (16).

In 2/20 BCEs, CK15 was detected gel-electrophoretically (Fig. 5b). Moll et al. (5) also described the existence of this CK in some cases of BCEs and of the ORS of the hair follicle, respectively. In our study, we could not detect CK 15 in ORS cells using 2-DE or immunoblot, obviously due to its inconsistent and slight expression.

MoAb RPN1165 stained 30% of BCE and inconsistently the outermost cells and more centrally located cells of the hair bulb. CK19 is expressed in simple epithelia and is thought to be related to pluripotential cells (28). Our findings are in accordance with the results of Habets et al. (27) and Bartek et al. (28), who also detected CK19 in BCE and ORS cells immunohistochemically. By means of SDS-PAGE, however, CK19 could neither be found in solid BCEs nor in the ORS of the hair follicle. While the reaction of BCE and ORS cells with RPN1165 was clearly positive, the amount of CK19 in BCE and ORS was too small to be detected by gel electrophoresis as in the cases of CK8. The presence of CK 19 suggests that populations of pluripotent and actively proliferating cells exist both in BCEs and in the lower ORS.

Based on our immunohistochemical and biochemical results, we thus conclude that the ORS below the isthmus may serve as a cellular pool for the generation of solid BCEs rather than the basal cell layer of human epidermis.

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