Hemidesmosomes, Collagen VII, and Intermediate Filaments in Basal Cell Carcinoma

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We have undertaken an analysis of hemidesmosomes (HD) and their associated structures, intermediate filaments (IF) and anchoring fibrils (AF), in various types of basal cell carcinoma (BCC). Using a combination of electron microscopy and immunofluorescence microscopy we show that there is a correlation between the loss of HD and tumor type (i.e., in solid and infiltrative BCC hemidesmosomes are present, sometimes in reduced numbers), while there appears to be a lack of hemidesmosomes in cells of sclerosing specimens. Moreover, even though there is a loss of cytoplasmic constituents of the HD in sclerosing forms of BCC, this is not the case with regard to collagen VII, a component of AF, which are normally associated with the extracellular side of the HD. Collagen VII is localized to the basement membrane zone of tumor cells in the absence of the cytoplasmic constituents of HD. Furthermore, deposits of collagen VII occur in the connective tissue close to tumor cell populations in all but one of the BCC specimens we analyzed. In addition to modifications in HD and AF in BCC tissue, there are changes in the cytoskeletal elements of both tumor cells and the normal appearing epidermis that overlies tumor areas. In sclerosing BCC microfilaments are commonly observed along the basal portions of tumor cells where they abut the connective tissue. IF are often found interacting with these microfilaments. Indirect immunofluorescence analysis of tumor tissue using a monoclonal keratin antibody preparation, AE1, which in normal epidermis stains basal cells, reveals that AE1 antibodies only weakly stain tumor cells. Moreover, in the epidermis that overlies tumor cell regions AE1 antibodies stain suprabasal cells and not basal cells. This change in staining pattern generated by AE1 antibodies appears to depend upon the proximity of tumor cells. These results are discussed in relation to the organization of the HD and its associated AF and IF. The possibility that HD, IF, and AF antibody preparations may be of diagnostic use is raised. *J Invest Dermatol 93:662–671, 1989*

Intermediate filaments (IF) are a major component of the cytoskeleton of mammalian cells [1–3]. In normal epithelial tissues IF are composed of keratins, a family of proteins ranging in molecular weight from 40 to 68 kD [4]. The precise composition of IF in an individual cell in an epithelial tissue depends upon the position of the cell within the tissue, the differentiated state of the cell, or the developmental stage of the cell [5,6]. Normal human epidermis contains a number of keratin polypeptides [4]. These keratins can be divided into either acidic or basic, the general rule appearing to be that one acidic keratin forms a pair with a basic keratin [7,8]. Monoclonal antibodies that recognize either human acidic or basic keratins, the former termed AE1 and the latter termed AE3, prepared by Sun et al have been used to analyze keratin expression in both normal epithelial tissues in certain hyperproliferative and non-hyperproliferative epithelial diseases [9].

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Abbreviations:
AF: anchoring fibril
BCC: basal cell carcinoma
BMZ: basement membrane zone
BP: bullous pemphigoid
HD: hemidesmosome
IF: intermediate filament
PBS: phosphate buffered saline

Along the basal surface of basal cells of normal human epidermis IF are found in association with hemidesmosomes (HD), cellular organelles considered to play an important role in the adherence of epithelial cells with the underlying connective tissue [10]. The composition of the HD remains obscure, although it has recently been shown that certain autoantibodies in the serum of patients with the blistering skin disease bullous pemphigoid recognize a HD plaque component [11–14]. Furthermore, anchoring fibrils (AF), which are associated with the connective tissue side of the HD, are composed of collagen VII [15]. Indeed it has been proposed that the HD-AF complex provides a structural link between the connective tissue and the IF network of the epithelial cell [16].

In this paper we have investigated the fate of certain components of the HD-AF complex and its associated IF in basal cell carcinoma (BCC). We show that there is a disruption of the HD-AF complex in BCC. Using the keratin antibody AE1 we show that there are not only modifications in the IF of BCC compared with normal basal epidermal cells but also changes in keratins in the normal appearing epidermis that overlies tumor cell populations.

MATERIALS AND METHODS

Antibodies The mouse monoclonals AE1 and AE3 (a gift from Dr. T-T. Sun) were used in this study. The human collagen VII monoclonal antibody preparation was a gift from Dr. Robert Burgeson [15]. A bullous pemphigoid serum sample was obtained from Dr. Robert Marder (Chief of the Clinical Immunology Laboratory, Northwestern Memorial Hospital, Chicago) and Dr. Ruth Frienkel (Department of Dermatology, Northwestern University Medical School). This serum sample contains autoantibodies that recognize

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a plaque component of the HD as determined by immunoelectron microscopy [14].

Specimens BCC tissue from 14 patients was used in this study. All tumors were from the head, neck, or scalp and were solitary and well circumscribed. The skin surrounding each tumor was injected with 1% lidocaine with epinephrine at a dilution of 1 : 200000 and a 3-4 mm diameter specimen with intact overlying epidermis was removed, immediately frozen in liquid nitrogen, and stored at −80°C. Tumors were classified as being predominantly solid (six specimens), infiltrative (three specimens), sclerosing (three specimens), solid with focal areas of sclerosis (one specimen), sclerosing with focal solid areas (one specimen), and infiltrative with focal solid areas (one specimen). Infiltrative BCC is a distinct histologic subtype recently characterized by Siegle et al [17]. Fourteen control non-tumor skin specimens were also obtained from these same patients and were frozen in liquid nitrogen. Portions of a solid, infiltrative BCC and two sclerosing BCC, as well as two control non-tumor tissue specimens, were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) and then processed for electron microscopy (see below).

Immunofluorescence Sections (3-5 μm thick) of frozen tissue were prepared on a Tissue-Tek cryostat and placed on slides. Sections were fixed for 5 min in −20°C acetone and air dried. Antibody preparations (hybridoma medium in the case of AE1, AE3 and the collagen VII antibody or bullous pemphigoid serum diluted 1 : 50 in PBS) were overlaid on the sections, and the slides were incubated for 45 min at 37°C. The slides were then extensively washed in PBS and sections were incubated in appropriate fluorescein conjugated secondary antibody (goat anti-mouse IgG for the monoclonal antibody preparations and goat anti-human IgG for the human serum sample). Sections were washed in PBS, mounted in gelvatol (Monsanto, St. Louis, MO), and covered with a glass coverslip. For double label immunofluorescence, bullous pemphigoid serum was added to hybridoma medium to a dilution of 1 : 50, and this mixture was overlaid on the sections. Following 45 min at 37°C, the sections were washed and then incubated for a further 45 min at 37°C in a mixture of fluorescein conjugated goat anti-mouse IgG and rhodamine conjugated goat anti-human IgG. Sections were washed and mounted in gelvatol as above. Stained sections were viewed using a Zeiss Photomicroscope III equipped with epifluorescence optics. Fluorescence micrographs were taken using Kodak Plus-X film. Films were developed in Diafine (Acufine Inc., Chicago, IL) two stage developer.

Conventional and Immunoelectron Microscopy For conventional electron microscopy the glutaraldehyde fixed specimens were post-fixed in 1% OsO_4 in PBS, rinsed in distilled water, and dehydrated and embedded as previously described [18]. For immunoelectron microscopy, cryostat sections, 3-5 μm thick, placed on slides were fixed for 5 min in −20°C acetone and air dried. Hybridoma medium was overlaid on the sections that were then incubated overnight at room temperature. After thorough washing, the sections were incubated in 5 nm gold conjugated goat anti-mouse IgG (Janssen Pharmaceutica Inc., Piscataway, NJ) for 6 h at room temperature. Following extensive washing, sections were fixed in 1% glutaraldehyde in PBS, postfixed in 1% OsO_4, and then dehydrated and embedded as detailed elsewhere [18]. The plastic embedded cryostat sections were removed from the glass slide following emersion of the slide in liquid nitrogen.

Thin sections of embedded material were prepared on a Reichert Ultracut E (Reichert Instruments, Buffalo, NY) using a diamond knife and were mounted on uncoated copper grids. The grids were stained with uranyl acetate and lead citrate. Thin sections were viewed in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 60 kV.

RESULTS

Immunofluorescence Analyses Cryostat sections of both normal skin and areas of human skin containing BCC were processed for indirect immunofluorescence using BP autoantibodies.

In cryostat sections of normal human skin BP autoantibodies generate a punctate stain along the basement membrane zone (BMZ) (Fig 1*a*) [11–14]. A similar distribution is also observed in the normal appearing epidermis that overlies tumor cell populations (Fig 1*b*). In the four BCC specimens containing sclerosing tumor areas, BP autoantibodies fail to stain the majority of areas of BCC cells where they abut the connective tissue (results not shown). Indeed, in certain instances there is an abrupt cessation of BP autoantibody staining around BCC cells that appear to be arising from the epidermis (Fig 1*b*). In the other 11 BCC specimens staining generated by BP autoantibodies around tumor cell populations is often incomplete, and intense staining is occasionally observed within or between the cancer cells (Fig 1*c*).

Because BP autoantibodies recognize a plaque component of the HD, the results described above suggest that there is a loss or partial loss of HD in BCC cells compared to normal basal cells.

A fluorescent line along the BMZ is observed in cryostat sections of normal human skin prepared for indirect immunofluorescence using an antibody preparation directed against collagen VII (Fig 2*a*). A similar distribution of collagen VII is also seen along the BMZ of the normal appearing epidermis that lies over tumor areas (Fig 2*b*). In all the BCC specimens the collagen VII antibody preparation produces a punctate stain around the groups of tumor cells (Fig 2*b,c*). Indeed, unlike normal skin, in all but one specimen large amounts of collagen VII reactive material, which has no obvious association with groups of epidermal cells, was also found scattered throughout the connective tissue (Fig 2*c*).

In order to compare the distribution of collagen VII and the plaque component of the HD that is recognized by BP autoantibodies, double label immunofluorescence was performed on cryostat sections of BCC specimens. In the epidermis overlying tumor regions there is a similar distribution of collagen VII and BP antigen (Fig 3*a,b*). However, collagen VII can be detected around sclerosing tumor cell populations which appear to lack BP antigen (Fig 3*a,b*). Furthermore, we were unable to detect BP antigen in association with the collagen VII containing deposits in the connective tissue of the tumor tissue we described above (Fig 3*a,b*).

In our analysis of the IF of BCC tissue we made use of two monoclonal antibody preparations, AE1 and AE3, which are directed against acidic and basic keratins, respectively. In cryostat sections of diseased tissue (i.e., solid, infiltrative and sclerosing BCC types) AE3 generates intense staining of both BCC cells and the cells of the epidermis that overlies the patches of tumor cells (Fig 4*b*). In contrast, cells of all the BCC we examined are only weakly stained by AE1 (Fig 5*b* shows a specimen containing a solid BCC; also see Fig 6). Moreover, in areas of normal appearing epidermis overlying tumor cells, the AE1 antibodies generate staining in the suprabasal cells and fail to stain the basal cells (Figs 5*b* and 6). Furthermore, the latter pattern appears to depend upon the proximity of the normal appearing epidermis to the areas of tumor. Staining of the suprabasal cells by AE1 antibodies is lost in epidermal cells that are located some distance from the tumor regions (Fig 6 shows a sclerosing BCC specimen).

Electron Microscopy One specimen of normal skin and four BCC specimens (one solid, one infiltrative, and two sclerosing BCC specimens) were fixed and processed for conventional transmission electron microscopy. In normal skin HD are located along the dermal-epidermal border (Figure 7*a*). A fully formed BMZ consisting of the lamina rara, densa, and lucida is evident (Fig 7*a*). Furthermore, AF are also seen along the connective tissue side of the BMZ (Fig 7*a*). In both the infiltrative and solid BCC specimens, either normal appearing HD or HD-like plaque structures similar to those described by McNutt [20] are located along the basal surface of tumor cells where they abut the connective tissue (results not shown). In contrast, in the sclerosing BCC specimen we studied, no obvious HD were observed in tumor cells abutting the connective tissue (Fig 7*b,c*) [20]. Moreover, the lamina densa of the BMZ of such BCC cells is either absent or incomplete (Fig 7*b*). Along the basal surface of BCC cells that interact with the connective tissue

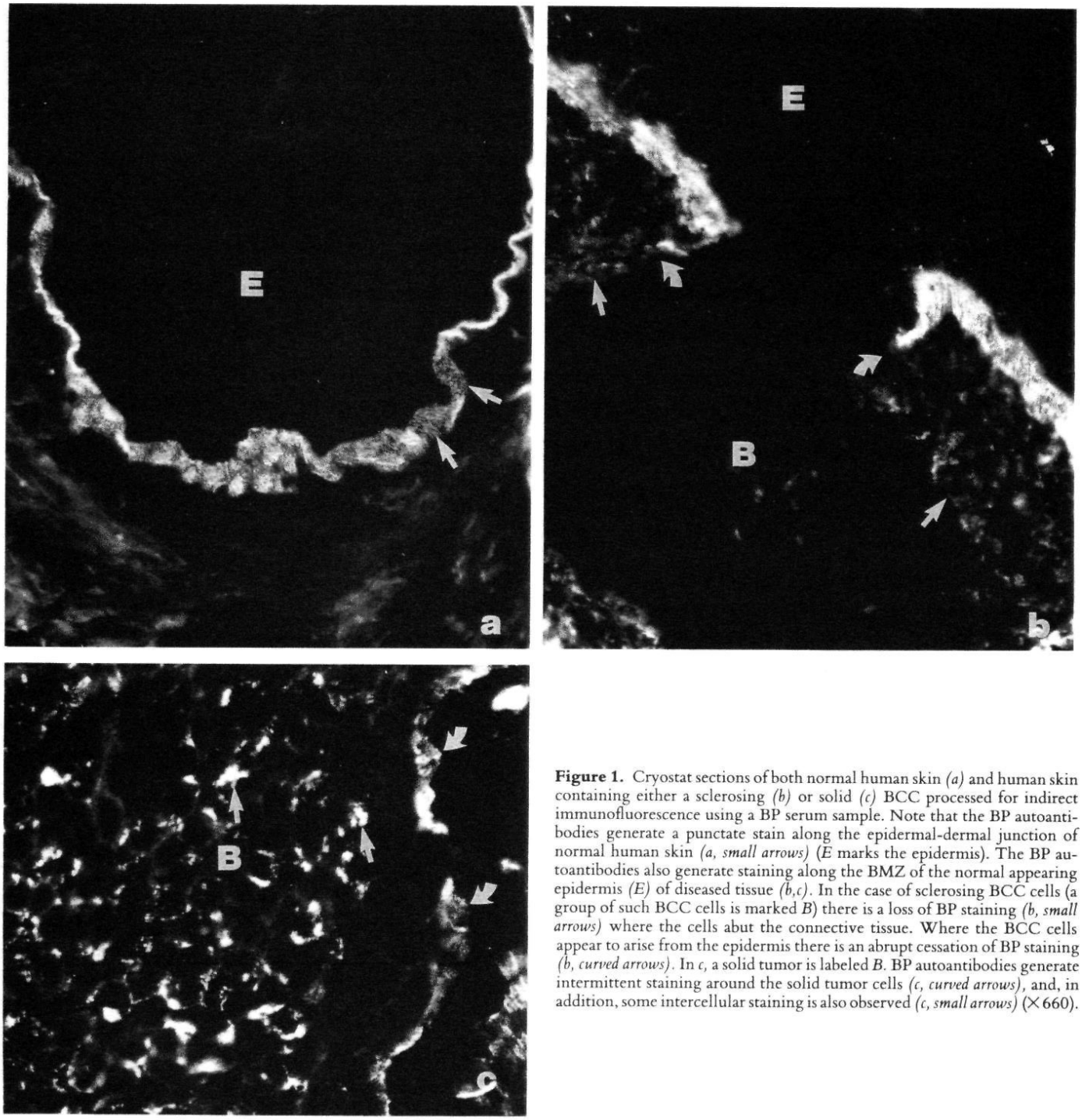

Figure 1. Cryostat sections of both normal human skin *(a)* and human skin containing either a sclerosing *(b)* or solid *(c)* BCC processed for indirect immunofluorescence using a BP serum sample. Note that the BP autoantibodies generate a punctate stain along the epidermal-dermal junction of normal human skin *(a, small arrows)* (*E* marks the epidermis). The BP autoantibodies also generate staining along the BMZ of the normal appearing epidermis *(E)* of diseased tissue *(b,c)*. In the case of sclerosing BCC cells (a group of such BCC cells is marked *B*) there is a loss of BP staining *(b, small arrows)* where the cells abut the connective tissue. Where the BCC cells appear to arise from the epidermis there is an abrupt cessation of BP staining *(b, curved arrows)*. In *c*, a solid tumor is labeled *B*. BP autoantibodies generate intermittent staining around the solid tumor cells *(c, curved arrows)*, and, in addition, some intercellular staining is also observed *(c, small arrows)* (×660).

there are often bundles of microfilaments oriented with their longitudinal axis parallel to the basal surface (Fig 7*c*). Strikingly, IF bundles can be observed in association with the microfilament bundles (Fig 7*c*). In normal epidermal basal cells microfilament bundles rarely, if ever, occur along the basal cell surface.

We were unable to observe AF around tumor cells by conventional electron microscopy (Fig 7*b,c*). However, our immunofluorescence analyses of BCC tissue reveals that collagen VII, a major component of AF [15], is present around tumor cells and also occurs scattered throughout the connective tissue of all but one of the diseased specimens we studied (Fig 2).

In normal human skin the collagen VII antibody preparation used in these studies localizes to the connective tissue side of the lamina densa of the BMZ, the region from which AF appear to arise (Fig 8*a*). In addition, certain electron amorphous bodies some distance from the BMZ are also recognized by the collagen VII antibodies (*inset,* Fig 8*a*). The latter appear to be attachment sites for AF in the connective tissue (*inset,* Fig 8*a*). These results confirm those presented by Sakai et al [15]. In the case of BCC tissue collagen VII is found in the lamina densa of the BMZ around tumor islands (Fig 8*b*). Furthermore, collagen VII is also present in amorphous bodies that occur throughout the connective tissue (Fig 8*c*). These appear similar to the AF attachment sites seen in normal skin (compare Fig 8*a* and *c*).

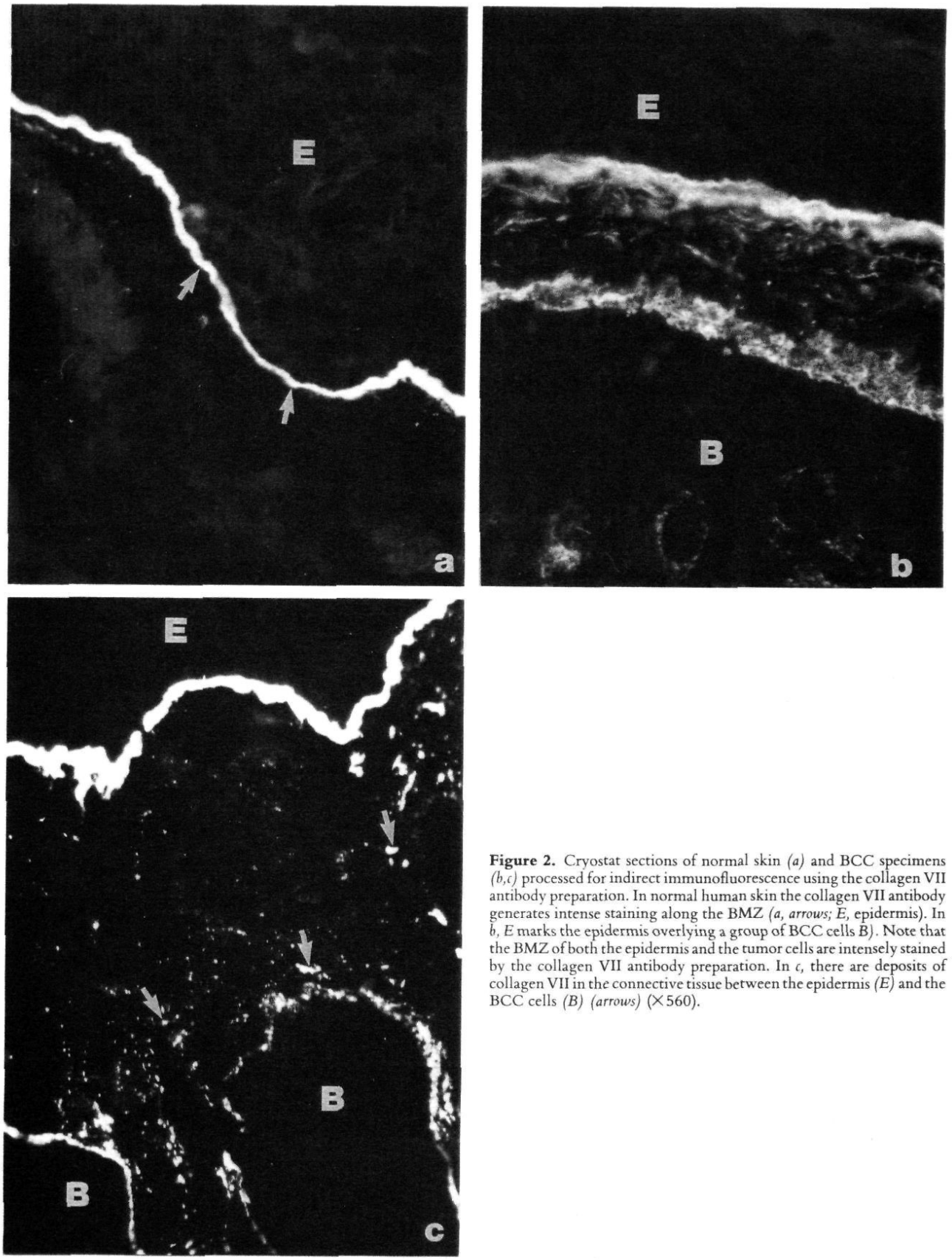

Figure 2. Cryostat sections of normal skin *(a)* and BCC specimens *(b,c)* processed for indirect immunofluorescence using the collagen VII antibody preparation. In normal human skin the collagen VII antibody generates intense staining along the BMZ *(a, arrows; E,* epidermis). In *b, E* marks the epidermis overlying a group of BCC cells *B)*. Note that the BMZ of both the epidermis and the tumor cells are intensely stained by the collagen VII antibody preparation. In *c,* there are deposits of collagen VII in the connective tissue between the epidermis *(E)* and the BCC cells *(B) (arrows)* (×560).

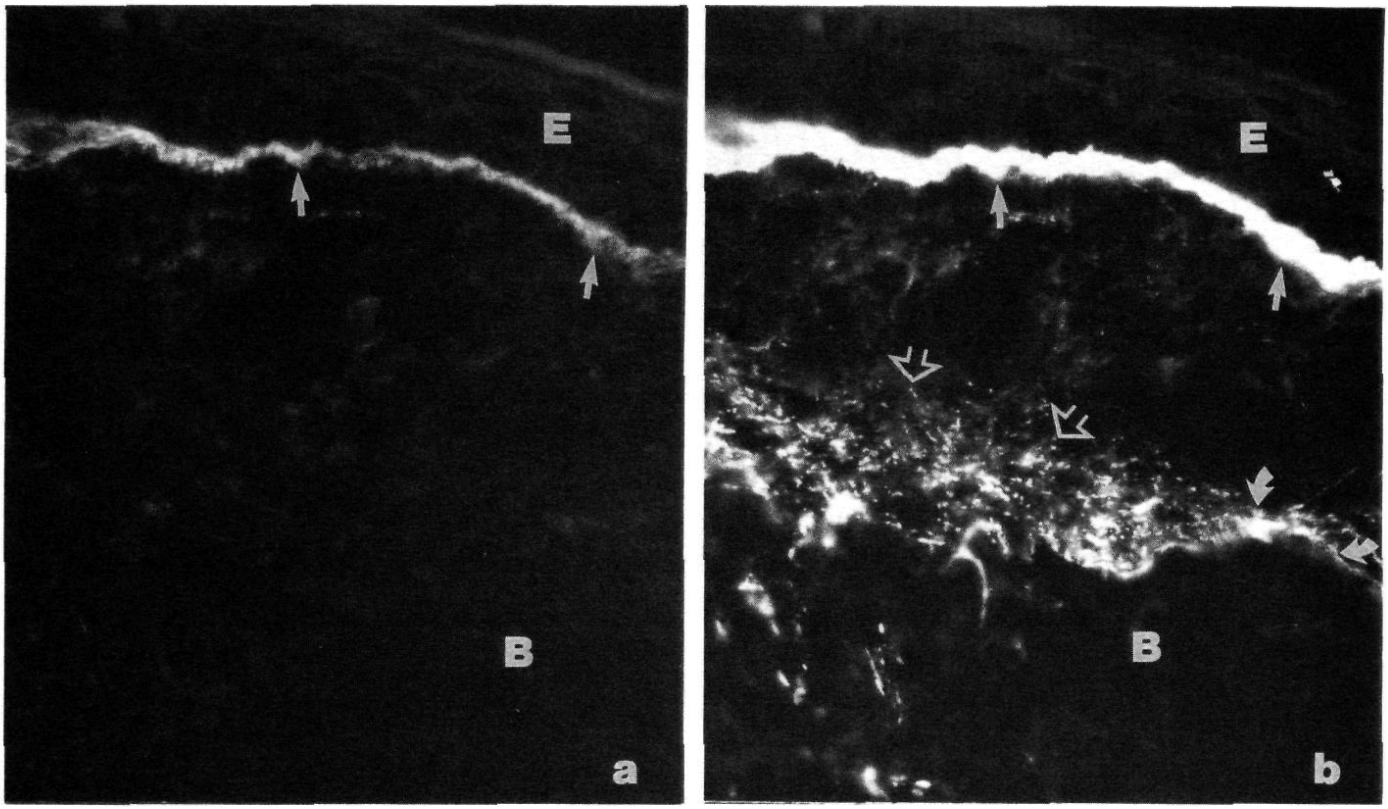

Figure 3. Double label immunofluorescence labeling of a cryostat section of BCC tissue using BP autoantibodies *(a)* and a collagen VII antibody preparation *(b)*. Note that both the BP autoantibodies and the collagen VII antibodies generate staining along the epidermal-dermal border *(a,b, small arrows)*. *E* denotes the epidermis that overlies tumor cells *(B)*. The collagen VII antibodies generate intense staining around the tumor cells *(b, curved arrows)* and in the connective tissue *(b, open arrows)*, while the BP autoantibodies do not (×560).

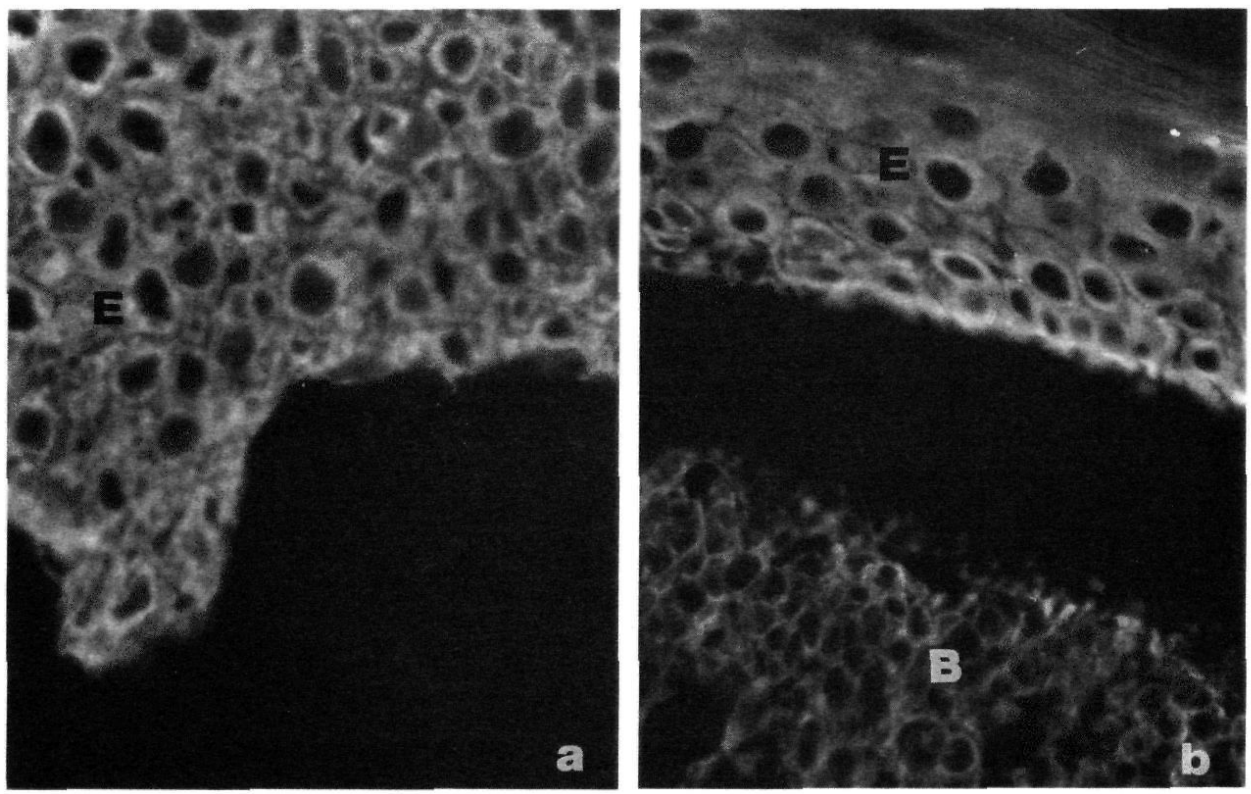

Figure 4. Cryostat sections of normal skin *(a)* and BCC tissue *(b)* processed for indirect immunofluorescence using the keratin monoclonal antibody AE3. The AE3 antibodies stain both normal epidermis *(E* in *a)* and the epidermis overlying tumor cells *(E* in *b)*. Furthermore, the AE3 antibodies stain BCC cells *(B* in *b)* (×560).

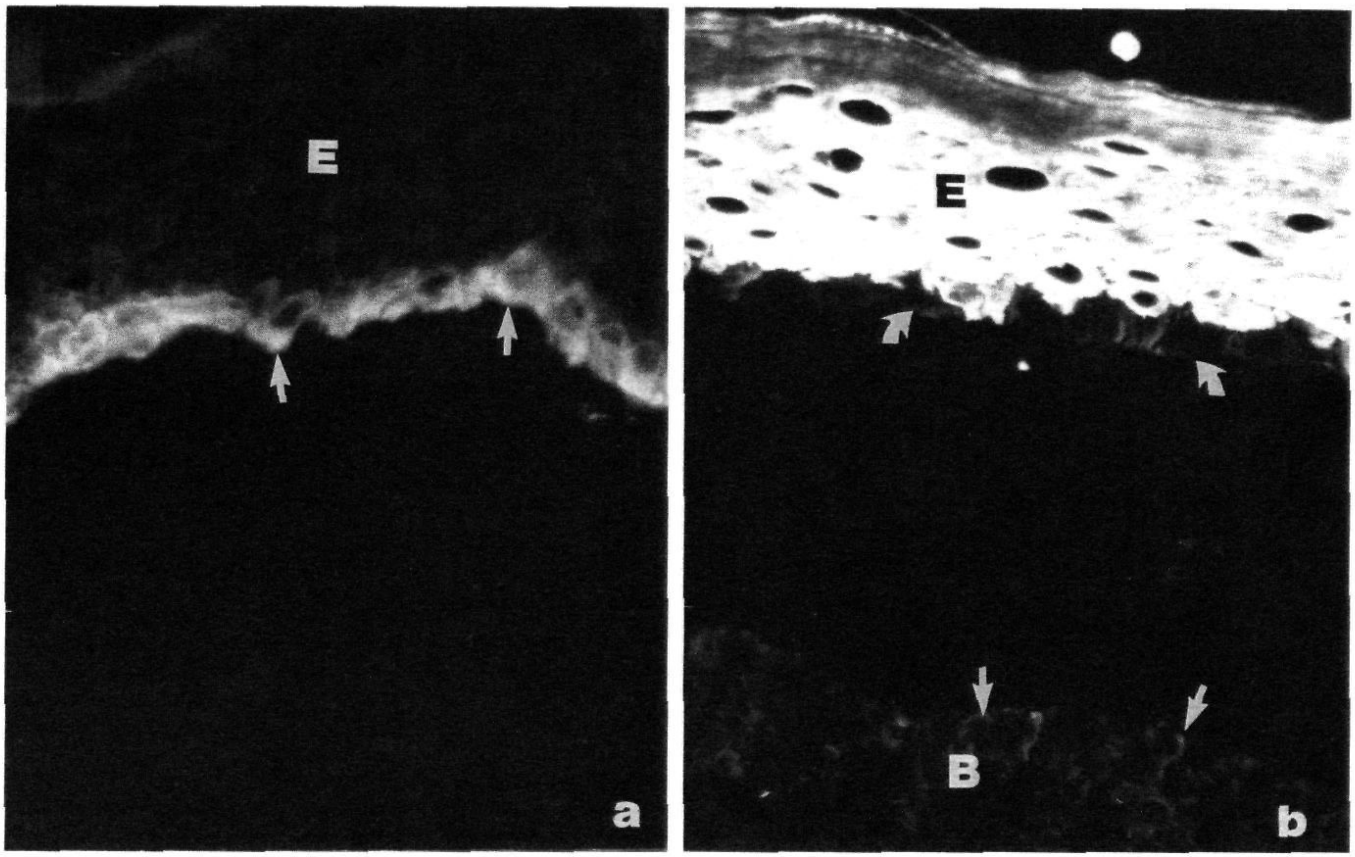

Figure 5. Cryostat sections of normal skin *(a)* and BCC tissue *(b)* processed for indirect immunofluorescence using the monoclonal antibody AE1. In normal skin AE1 antibodies stain the basal cells of the epidermis *(E) (a, arrows)*. AE1 antibodies only weakly stain certain cells in the BCC region *(b, small arrows)*, whereas in the epidermis overlying the tumor cells the AE1 antibodies stain the suprabasal cells and do not stain the basal epidermal cells *(b, curved arrows)* (×560).

DISCUSSION

Our immunofluorescence and electron microscopic results indicate HD appear normal in the epidermis that lies above the tumor cell populations. However, there is an obvious loss of HD in the sclerosing BCC cells, as determined electron microscopically, confirming the results of McNutt [20]. In the solid and infiltrative forms of BCC electron microscopy reveals that there is partial loss of HD [20]. In sclerosing BCC specimens there is a loss of BP autoantibody staining around tumor cells, whereas in the solid and infiltrative BCC specimens there is patchy BP autoantibody staining in the BMZ of tumor cells. These results using the BP autoantibodies extend observations by Stanley et al [21]. The latter detected either no or faint and discontinuous BP staining of the BMZ surrounding tumor cells in 13 BCC specimens. However, these authors did not categorize their BCC specimen types and thus were unable to relate their results to the type of BCC specimen that they were studying.

While there is a loss of the cytoplasmic constituents of the HD, this does not appear to be the case with regard to collagen VII, a component of the AF that are associated with the extracellular side of the HD. Although ultrastructural analyses of four BCC specimens reveals that no obvious AF occur in association with tumor cells, collagen VII appears around tumor cell populations in all the BCC specimens we studied, even in the absence of BP autoantibody staining. This result appears to contradict Lane et al [22], who reported that there is a decrease in AF antigens in BCC. Furthermore, the AF, or more specifically collagen VII containing elements of AF, do not appear to be able to act as nucleating sites for HD formation, at least in the BCC cells, as suggested by Gipson et al [23]. The inability of AF to induce HD assembly in BCC cells may, for example, result from a lack of the cytoplasmic constituents of HD in tumor cells. Alternatively, the mechanism of HD formation in the BCC cells may be aberrant, or the cell surface components with which the AF interact and which trigger HD formation may be altered or lost in BCC cells.

In all but one of the BCC specimens we detected deposits of collagen VII in the connective tissue close to tumor regions but with no obvious direct connection with tumor cells. Sakai et al [15] reported that collagen VII is produced by epithelial cells. Thus we speculate that the collagen VII deposits are produced by tumor cells as they migrate through the connective tissue.

In addition to the changes in HD in BCC cells there also appear to be organizational modifications in the cytoskeletal elements of tumor cells. In the sclerosing type of BCC there is an accumulation of microfilaments along the basal surface where the tumor cells interact with the connective tissue. This phenomenon is not peculiar to BCC tissue because microfilaments are also found in this location in normal basal keratinocytes in certain instances; for example, in embryonic skin [24]. McNutt [20] has suggested that in BCC this microfilament accumulation is related to the enhanced motility of invasive carcinoma cells. We also observed that IF appear to interact with these microfilaments in a similar manner to that recently described in cultured mouse keratinocytes maintained in low calcium medium [25]. Moreover, the distribution of microfilaments along the cell surface and the enclosure of IF by these microfilaments is also remarkably similar to that seen in the low calcium cultured keratinocytes [25]. Interestingly, it has been shown, using time-lapse video, that keratinocytes cultured in low calcium medium are more motile than their counterparts in normal medium [26]. Thus the similarity in the organization of the cyto-

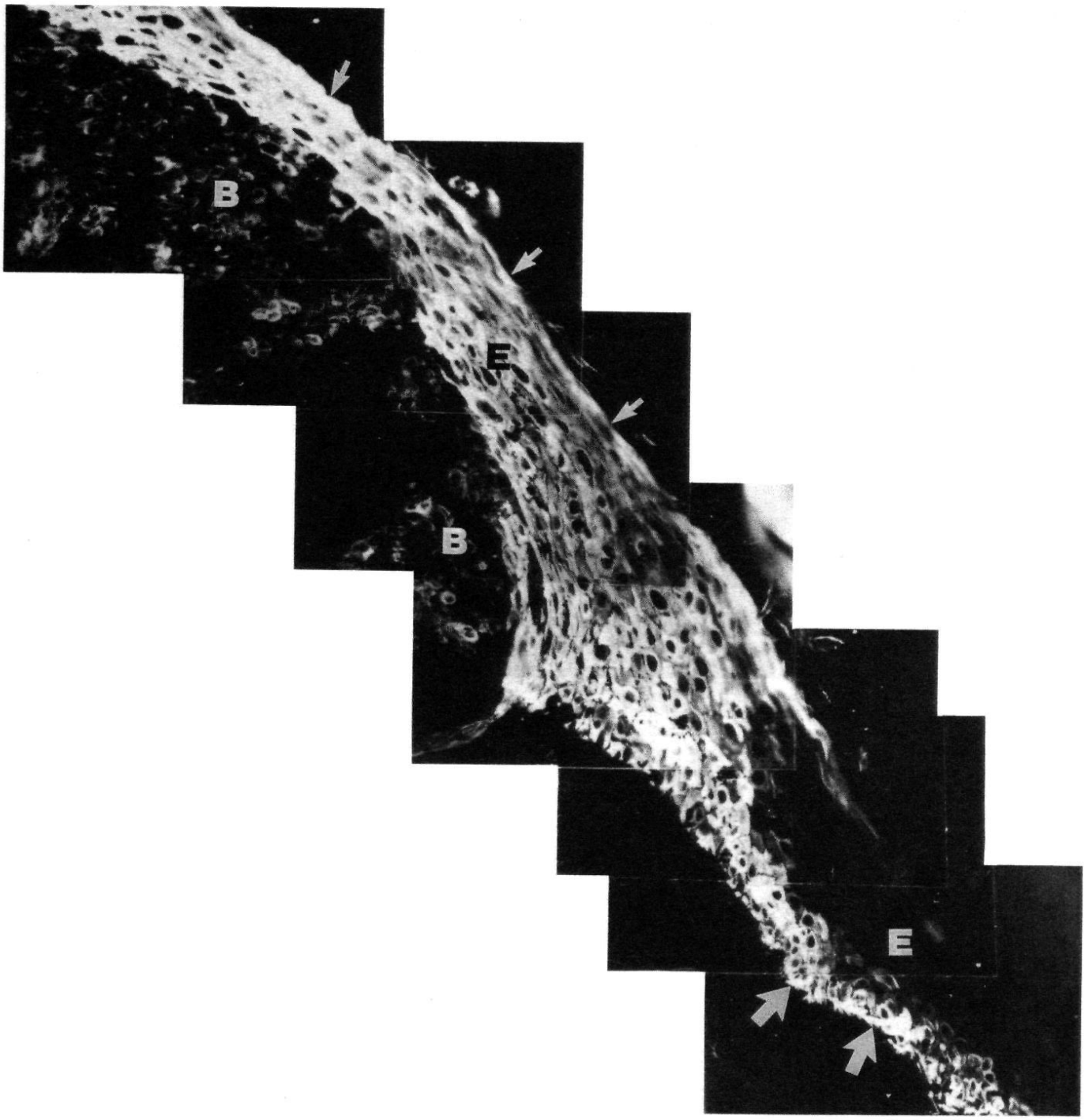

Figure 6. A cryostat section of a sclerosing BCC specimen processed for indirect immunofluorescence using the AE1 antibody preparation. Note that the AE1 antibodies stain the basal epidermal cells in the normal appearing epidermis *(E)* some distance from the tumor cell areas *(large arrows)*, whereas in the epidermis immediately overlying the tumor cells *(B)* the AE1 antibodies intensely stain suprabasal cells *(small arrows)* (×220).

skeletal elements of BCC cells and the low calcium cultured keratinocytes may well reflect their motile behavior.

There are also modifications in the immunologic properties of IF in BCC cells compared with normal basal epidermal cells. Such findings correlate with previous biochemical studies, which show that there are changes in keratin expression in BCC cells compared to their normal counterparts [27,28]. Our study reveals that the monoclonal antibody AE1, which stains basal cells in normal epidermis, only weakly stains BCC cells. Furthermore, in the epidermis that overlies tumor areas, AE1 fails to stain basal cells but instead stains the suprabasal cells intensely [29,30]. This phenomenon is common to various conditions that show epidermal hyperproliferation, e.g., psoriasis, verruca, and seborrhic keratosis [29], and appears to be the result of an induction of keratin 16 [31].

The observation that the staining pattern generated by AE1 antibodies in the epidermis overlying BCC areas appears to depend upon the proximity of tumor cells suggests that tumor cells in some way influence the properties of normal tissues in their vicinity. The reason for this change in the keratin expression program is not understood. However, it is tempting to speculate that some type of

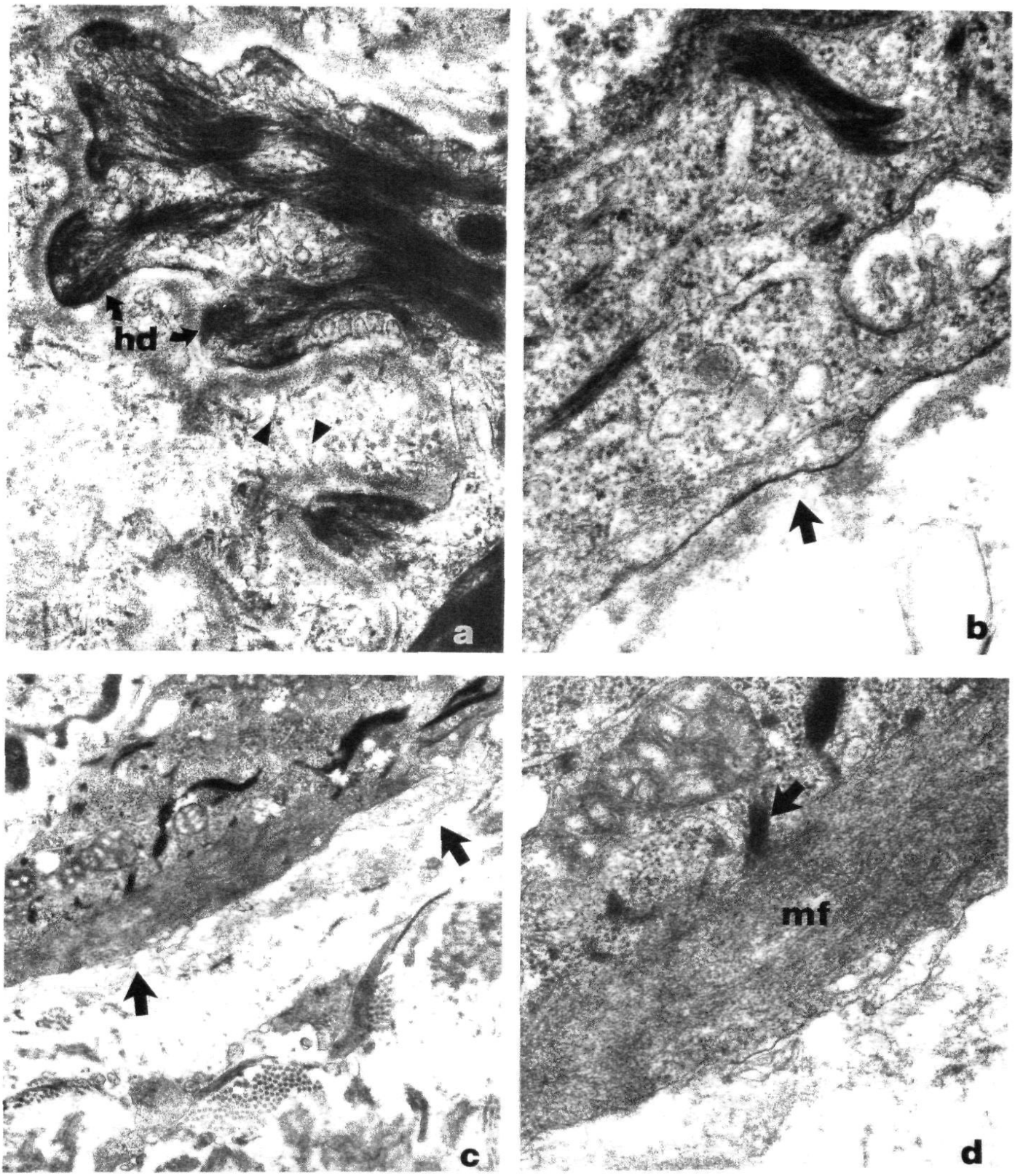

Figure 7. Electron micrographs of thin sections of normal human skin *(a)* and sclerosing BCC cells *(b,c,d)*. In normal skin hemidesmosomes are located in basal epidermal cells along the epidermal-dermal border *(hd* in *a)*. Note also the typical BMZ and anchoring fibrils *(arrowheads)* underlying the hemidesmosomes. Where the BCC cells abut the connective tissue no obvious hemidesmosomes or anchoring fibrils can be observed *(b,c)*, and the BMZ appears patchy *(b,c arrows)*. The micrograph shown in *d* is an enlargement of *c* to show that microfilaments *(mf)* occur along the basal surface of the BCC cell. Note that bundles of IF are found in association with the microfilaments *(c, arrow)* (a,b: ×60,000; c: ×15,000; d: ×60,000).

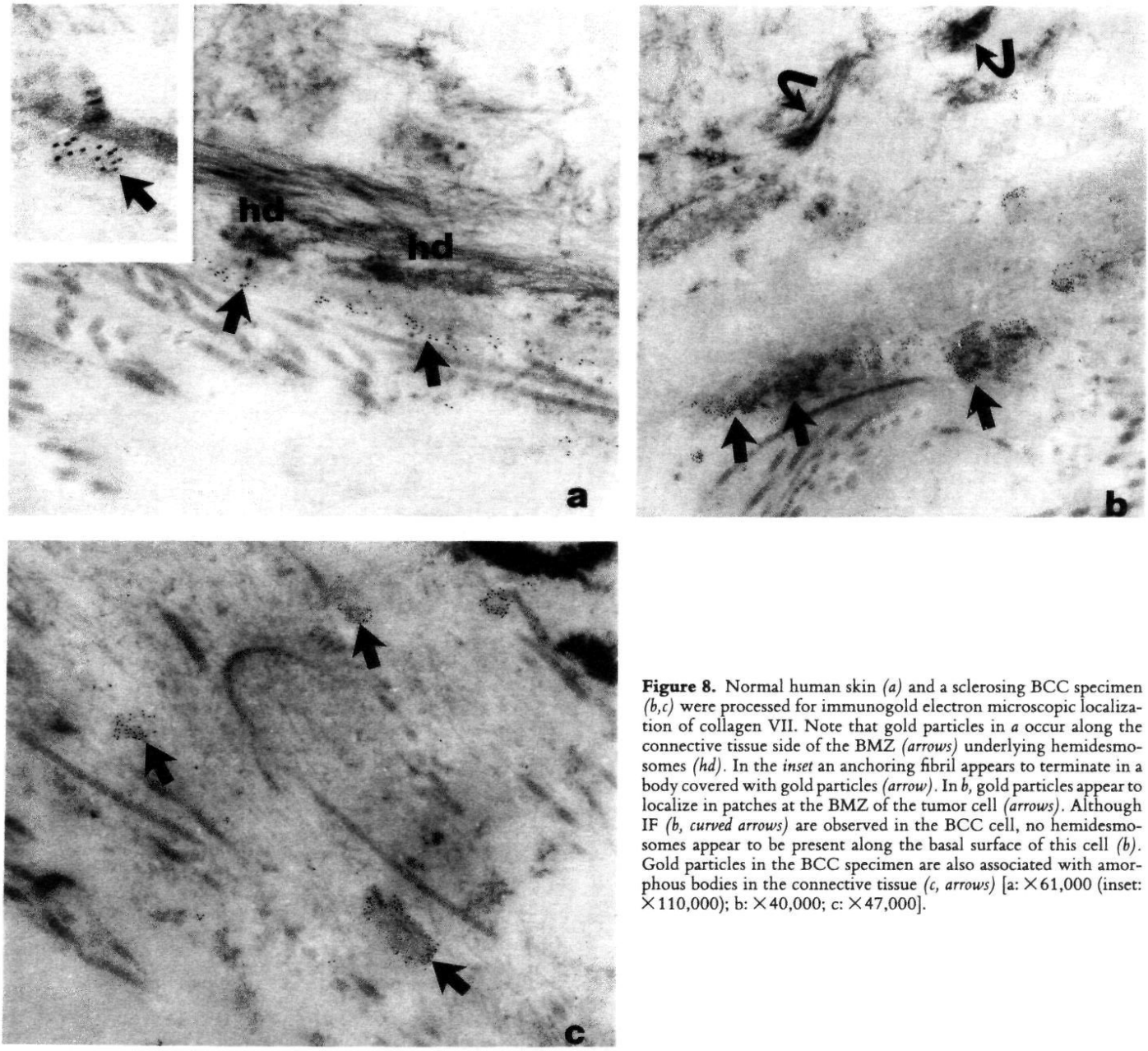

Figure 8. Normal human skin *(a)* and a sclerosing BCC specimen *(b,c)* were processed for immunogold electron microscopic localization of collagen VII. Note that gold particles in *a* occur along the connective tissue side of the BMZ *(arrows)* underlying hemidesmosomes *(hd)*. In the *inset* an anchoring fibril appears to terminate in a body covered with gold particles *(arrow)*. In *b*, gold particles appear to localize in patches at the BMZ of the tumor cell *(arrows)*. Although IF *(b, curved arrows)* are observed in the BCC cell, no hemidesmosomes appear to be present along the basal surface of this cell *(b)*. Gold particles in the BCC specimen are also associated with amorphous bodies in the connective tissue *(c, arrows)* [a: ×61,000 (inset: ×110,000); b: ×40,000; c: ×47,000].

signal is generated by the tumor cells in the connective tissue. This signal is then transmitted to the overlying epidermis, directly or indirectly inducing changes in the cytoskeleton. We are currently assessing the possibility that keratin and collagen in antibody preparations may be useful in analyses of tissue specimens from BCC patients in order to more precisely define the extent of tumor tissue.

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