BM-40 (Osteonectin, SPARC) Is Expressed Both in the Epidermal and in the Dermal Compartment of Adult Human Skin

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BM-40 (Osteonectin, SPARC) is the most abundant glycoprotein secreted by human osteoblasts. *In situ* hybridization studies on the expression of BM-40 mRNA in murine tissues have demonstrated the highest levels of transcripts in bone, but expression was also observed in several other mesenchymal tissues. In contrast, little is known about the expression of BM-40 in human tissues, especially in skin. Total RNA obtained from normal human skin was analyzed by northern blotting and revealed a marked expression of BM-40. To analyze its expression *in vivo*, *in situ* hybridization was performed, demonstrating that BM-40 is expressed in fibroblasts, smooth muscle, and endothelial cells in the dermis. Interestingly, BM-40 mRNA was also detected throughout the basal, spinous, and granular layers in the epidermis of adult human skin. Further analysis by immunohistochemistry revealed a marked deposition in the dermis that was most intense directly below the basement membrane in the papillary dermis and around vascular as well as glandular structures. In the epidermis, BM-40 protein could be detected intercellularly in suprabasal layers. This finding is further supported by the intercellular deposition of BM40 detected by immunofluorescence in cultured keratinocytes. This study demonstrates that BM-40 that has previously been thought to be exclusively expressed in extracellular matrix producing cells may in fact play a role in differentiation and maintenance of the epidermis. *Key words: epidermis/extracellular matrix/keratinocyte. J Invest Dermatol 110:122–126, 1998*

The glycoprotein BM-40 (also termed SPARC, osteonectin) is a secreted protein widely distributed in human and murine tissues. It has been identified in bone, where it is one of the major noncollagenous glycoproteins, in basement membranes, and in several other extracellular tissues (Termine *et al*, 1981; Dziadek *et al*, 1986; Sodek, 1993). Several studies describe high affinity binding to calcium (Engel *et al*, 1987; Maurer *et al*, 1992) and calcium-dependent binding to various collagens (Domenicucci *et al*, 1988; Sage *et al*, 1989b; Mayer *et al*, 1991; Nischt *et al*, 1991; Kelm and Mann, 1991), as well as binding to hydroxyapatite (Romberg *er al*, 1985) and platelet-derived growth factor (Raines *et al*, 1992) *in vitro*. In several cell culture studies using fibroblasts and endothelial cells, BM-40 has been shown to influence the control of cell shape, migration, growth control, and gene expression (for review, see Lane and Sage, 1994; Reed and Sage, 1996).

Even though the exact biologic function is still unknown, BM-40 is expressed at high levels during tissue repair, differentiation, mouse development, and growth, where it may act as an anti-adhesive factor for cells (Holland *et al*, 1987; Sage *et al*, 1989a; Sage and Bornstein, 1991; Tremble *et al*, 1993; Reed *et al*, 1993). This suggests a versatile function of BM-40 in the regulation of tissue synthesis and turnover, including a major role as a morpho-regulatory element. Although information regarding the structure and potential biologic functions of BM-40 has increased significantly over the last few years, relatively little is known about the expression and distribution of BM-40 in human tissues *in vivo*.

Therefore the aim of this study was to investigate BM-40 expression and synthesis in adult human skin. We report here that BM-40 is expressed in adult human skin in both the dermal and the epidermal compartment, indicating a new role for BM-40 in epidermal differentiation and growth *in vivo*.

MATERIALS AND METHODS

Tissue samples Normal skin was obtained from patients admitted for tattoo excisions following informed consent. Specimens were bisected with one half of the specimen undergoing routine paraffin processing and the other half being snap frozen in liquid nitrogen and stored at −70°C until required.

Cell culture Human skin fibroblasts were obtained from normal human skin using the explant culture technique. Primary keratinocyte cultures and subcultures were established according to the method of Limat *et al* (1989). The HaCaT cell line had been generously provided by Dr. N. Fusenig (DKFZ, Heidelberg). HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown to confluency in monolayer cultures in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum, 50 mg ascorbate per ml, 300 mg glutamine per ml, 50 mg streptomycin per ml, and 400 U penicillin per ml. Thereafter cells were either subjected to RNA isolation or grown on glass slides for subsequent immunohistochemical analysis.

Northern blot analysis Frozen skin biopsies were cut on dry ice and homogenized in 4 M guanidine-isothiocyanate containing 0.1 M 2-mercaptoethanol. Alternatively cells were scraped from cell culture dishes using a rubber policeman and dissolved in the same buffer. RNA was isolated by ultracentrifugation over a cesium chloride cushion according to published protocols (Maniatis *et al*, 1982). For northern blot analysis 5 mg total RNA was

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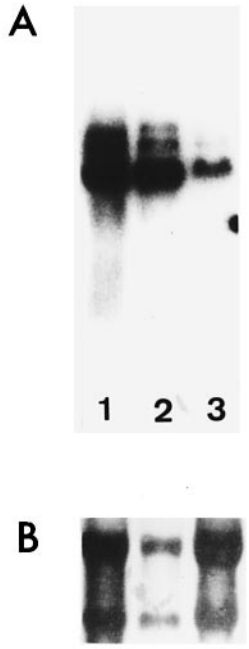

Figure 1. Comparison of BM-40 mRNA expression *in vivo* and *in vitro*. (*A*) Total RNA isolated from normal skin (*lane 1*; 5 mg), fibroblasts (*lane 2*; 2 mg), and keratinocytes (*lane 3*; 5 mg) was separated on a 1% agarose gel, blotted onto Genescreen, and hybridized with a ^{32}P-labeled random primed BM-40 cDNA probe as described in *Materials and Methods*. Hybridization revealed strong signals in all three samples. (*B*) Methylen blue staining is shown to confirm integrity and transfer of the RNA samples.

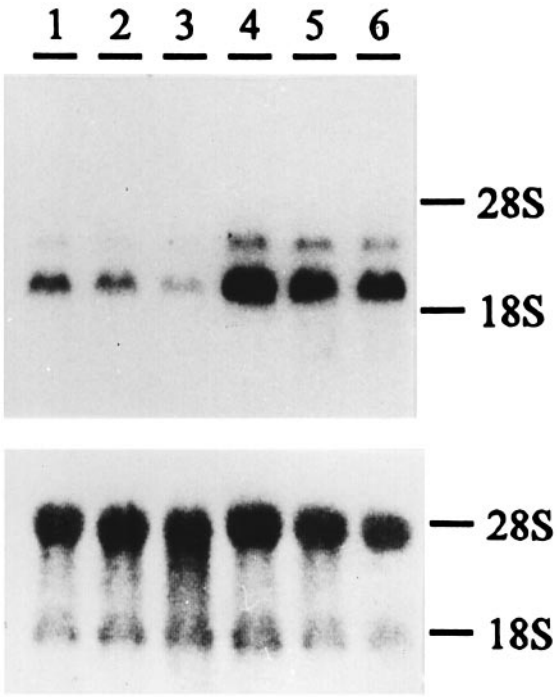

Figure 2. Increased BM-40 mRNA levels in keratinocytes after induction of differentiation. To induce differentiation in cultured keratinocytes the cells were cultivated in 3 mM sodium n-butyrate (*lanes 4–6*). Untreated keratinocytes were used as controls (*lanes 1–3*). For RNA isolation cells were harvested after 24 h (*lanes 1*, *4*), 48 h (*lanes 2*, *5*), and 72 h (*lanes 3*, *6*). The RNA samples were separated in a 1% agarose gel, blotted onto Genescreen, and hybridized to ^{32}P-labeled BM-40 cDNA as described in *Materials and Methods*. In the lower part methylen blue staining of rRNA is shown to confirm even loading of the RNA samples.

separated by gel electrophoresis in 1% agarose under denaturing conditions and then blotted onto a nylon membrane (Genescreen, Dupont, Boston, MA). As a control for the amount and integrity of RNA blotted, the membrane was stained with methylene blue, 0.04% in 0.2 M sodium acetate (pH 5.2). Filters were hybridized according to published protocols with ^{32}P-labeled random primed BM-40 cDNA probe. After hybridization filters were washed and exposed at −80°C to a radiosensitive film (Kodak, X-Omat AR, Rochester, NY).

***In situ* hybridization** Frozen 5 mm sections were investigated using the nonradioactive *in situ* hybridization technique with digoxigenin labeled oligonucleotides. The following anti-sense and sense 5′ digoxigenin labeled oligonucleotides corresponding to nucleotide position 136–169 were used (Lankat-Buttgereit *et al*, 1988): anti-sense, 5′-gctcccacagatacct-cagtcacctctgccacag-3′; sense, 5′-Ctgtggcagaggtgac-tgaggtatctgtgggag-3′. The specificity of these oligonucleotides was confirmed by northern blot analysis of fibroblast total RNA (not shown). Briefly, frozen sections were mounted on silane-coated slides and fixed with 4% paraformaldehyde in phosphate-buffered saline for 5 min and treated with 2 mg glycine per ml for 15 min thereafter. Prehybridization and hybridization were performed at 42°C in a mixture containing 6 × sodium chloride/sodium citrate, 1 × Denhardts solution, 100 mg tRNA per ml, 0.25% sodium dodecylsulfate. After hybridization and washing in 6 × sodium chloride/sodium citrate at 42°C, digoxigenin-labeled probes were visualized as described in the manufacturer's protocol (Boehringer, Mannheim, Germany). Alternatively, anti-sense and sense digoxigenin labeled riboprobes were prepared using a 490-bp BM-40 cDNA fragment subcloned into the vector pBluescript KS+ (Stratagene, La Jolla, CA) (Lankat-Buttgereit *et al*, 1988). The probes were labeled with digoxigenin 11-UTP using a RNA labeling kit (Boehringer) and hybridized in the same mixture with 50% formamide. After stringent washings, sections were counter-stained with eosin or methyl green and mounted.

Immunohistochemical staining Alkaline phosphatase anti-alkaline phosphatase staining was performed following established procedures (Schaumburg-Lever, 1987). Rabbit anti-mouse immunoglobulins and alkaline phosphatase anti-alkaline phosphatase complexes were obtained from Dako (Hamburg, Germany). The alkaline phosphatase reaction was demonstrated by incubation in a solution containing Fast Red TR (1 mg per ml) and naphtol AS-TR phosphate (0.2 mg per ml) (Sigma, St. Louis, MO). Levamisole (0.24 mg per ml) was added to block endogenous alkaline phosphatase activity.

Primary keratinocytes, HaCaT, and HeLa cells grown on glass slides were stained using fluoroscein isothiocyanate conjugated goat anti-IgG antibodies

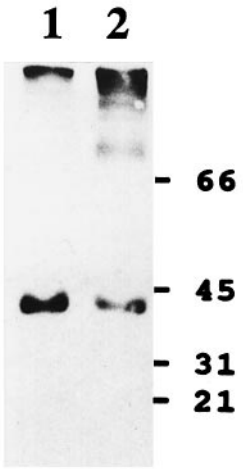

Figure 3. Secretion of BM-40 protein by primary keratinocytes and fibroblasts. Conditioned media from keratinocytes and fibroblasts were analyzed by immunoblotting. The proteins were precipitated from the media by trichloracetic acid and separated on a 10% sodium dodecylsulfate/polyacrylamide gel under reducing conditions, transferred to nitrocellulose, and incubated with BM-40 anti-serum as described in *Materials and Methods*. In *lane 1* 0.5 ml of fibroblast conditioned medium was applied, and in *lane 2* 2 ml of keratinocyte conditioned medium was applied. Calibration shown on the right was with globular protein (kDa). BM-40 is detected as a single band of about 42 kDa. The higher molecular bands are due to BM-40 aggregates sometimes observed with cultivated cells.

following established protocols. In brief, after incubation with 1% normal goat serum/1% bovine serum albumin in phosphate-buffered saline at room temperature, the slides were incubated with the primary polyconal rabbit anti-serum (Nischt *et al*, 1991) (diluted 1:400 in 1% normal goat serum/1% bovine

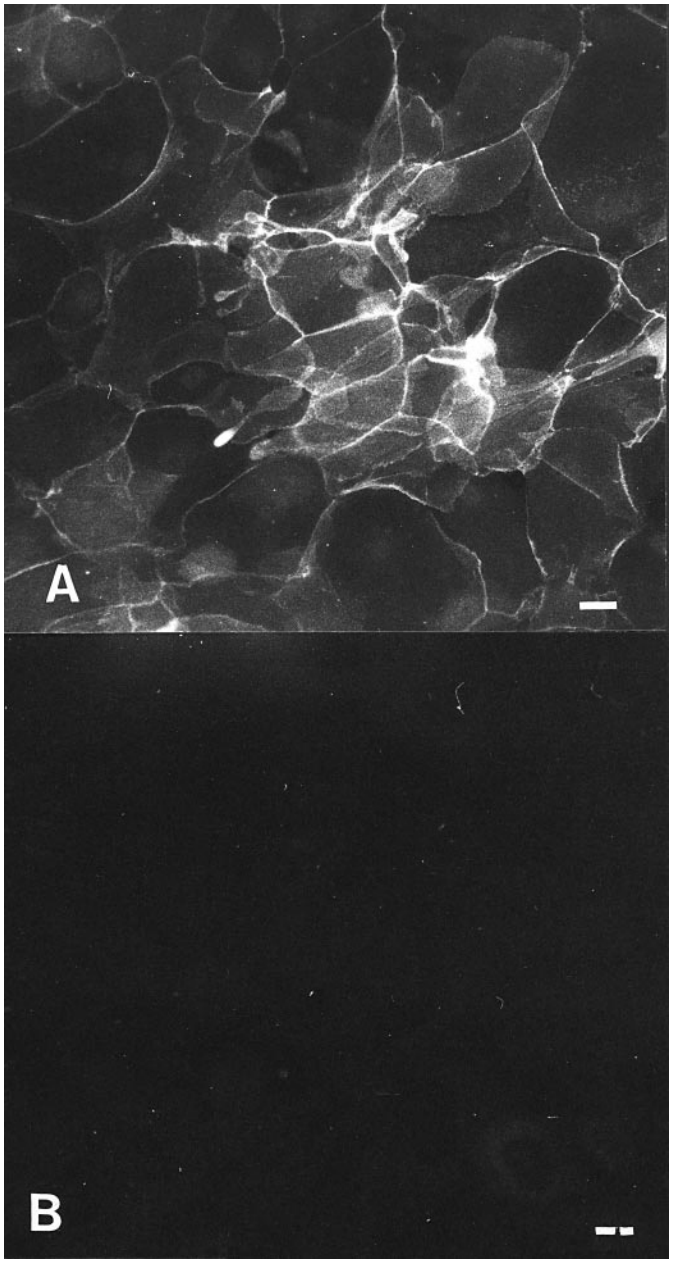

Figure 4. BM-40 is localized in the periphery of keratinocytes with suprabasal morphology. Primary human keratinocytes were grown on glass slides for 72 h. The cells then were stained without fixation by immunofluorescence with BM-40 anti-serum as described in *Materials and Methods*. (*A*) Note the fluorescence at the cell–cell contact sites of keratinocytes with suprabasal morphology; (*B*) staining of He La cells is shown as a negative control. *Scale bars*, 35 μm.

serum albumin in phosphate-buffered saline) for 45 min. Incubation with the secondary antibody (fluoroscein isothiocyanate conjugated goat anti-rabbit 1:400 in the same buffer) was carried out for 20 min. After washing and mounting fluorescence microscopy was performed. Negative controls consisted of the omission of primary antibodies, use of preimmune rabbit serum, and staining with the dye alone. As a positive control a polyclonal antibody directed against keratin was used. The stained sections were reviewed by two independent observers.

Western blot analysis Trichloracetic acid precipitable proteins from serum free culture medium (primary fibroblasts and keratinocytes) were resolved on a 10% sodium dodecylsulfate/polyacyrylamide gel under reducing conditions. Thereafter proteins were transfered to nitrocellulose (Amersham, Braunschweig, Germany). To block nonspecific antibody binding, the membrane was incubated overnight in tris buffered saline containing 5% skimmed milk powder. Subsequently the membrane was incubated with the polyclonal rabbit-anti-BM-40

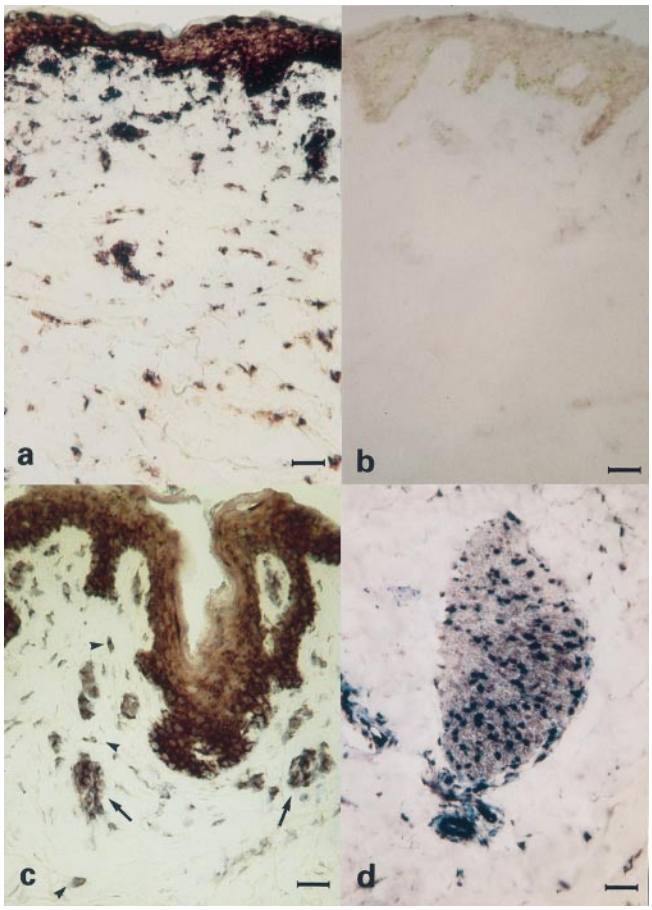

Figure 5. Nonradioactive *in situ* hybridization of normal adult human skin with BM-40 anti-sense and sense probes. *In situ* hybridization was performed on consecutive skin sections using digoxigenin labeled oligonucleotides. In (*a*) a section of normal human skin is shown after hybridization with the anti-sense oligonucleotide, whereas in (*b*) the section is shown after hybridization with the sense oligonucleotide (*scale bars*, 100 μm). Note that BM-40 mRNA is present in the basal and suprabasal cell layers of the epidermis. In (*c*) a higher magnification is shown. In the dermis BM-40 expression is found in fibroblasts (*arrowheads*) and perivascular areas (→) (*scale bar*, 50 μm); in (*d*) BM-40 expression in smooth muscle cells is shown (*scale bar*, 35 μm).

anti-serum diluted 1:2000. After washing, the membrane was incubated with an horseradishperoxidase-conjugated swine anti-rabbit antibody (Dako) followed by development using the ECL-system (Amersham).

RESULTS

Northern blot analysis of BM-40 in human skin compared with cultured fibroblasts and keratinocytes It is known from several *in vitro* studies that fibroblasts and keratinocytes are expressing BM-40 mRNA (Howe *et al*, 1990; Lankat-Buttgereit *et al*, 1991; Wrana *et al*, 1991; Ford *et al*, 1993). As BM-40 expression is usually induced when attachment-dependent cells are grown on plastic, a phenomenon referred to as "culture shock" (Sage *et al*, 1986; Lane and Sage, 1994), we were interested to compare BM-40 mRNA levels in skin directly with the levels expressed by keratinocytes and fibroblasts grown on plastic. As shown in **Fig 1**, northern blot analysis revealed the usual hybridization pattern for BM-40 mRNA in both fibroblasts and keratinocytes, with a high level of the 2.2 kb and a lower level of the 3 kb transcript that contains additional 3′ untranslated sequences (Swaroop *et al*, 1988); however, in keratinocytes the level was markedly lower when compared with fibroblasts (**Fig 1**, *lanes 2* and *3*). The signal obtained with RNA isolated from skin biopsies is very strong, indicating that the signals obtained with cultured keratinocytes and fibroblasts reflect the BM-40 mRNA level *in vivo*, although we cannot distinguish the cellular source of BM-40 mRNA in this experiment.

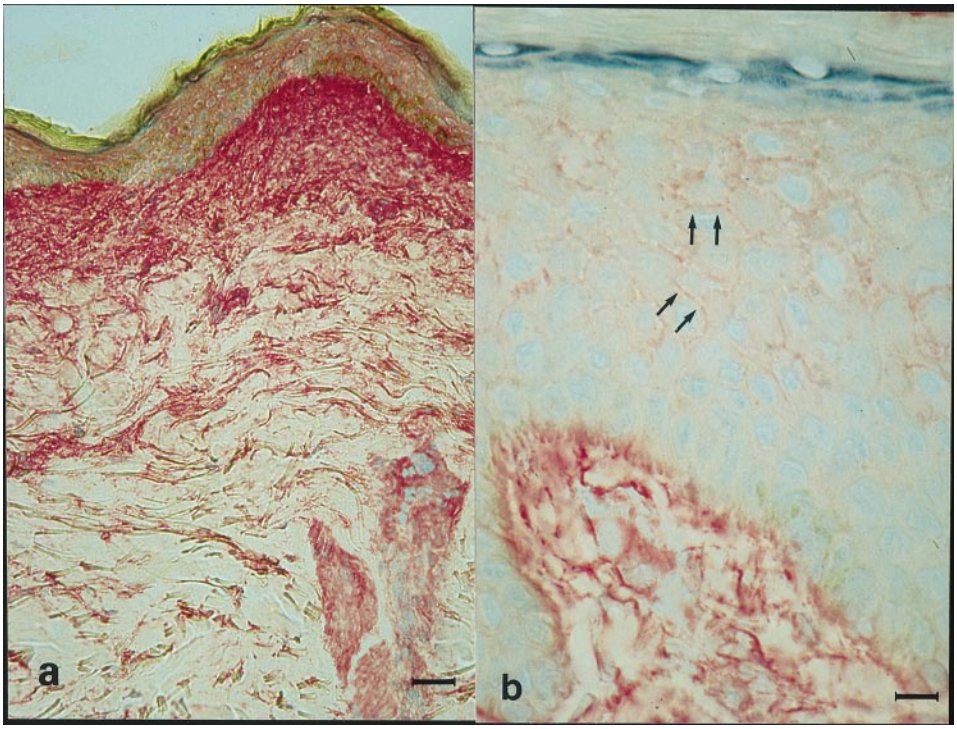

Figure 6. Immunohistochemistry showing BM-40 deposition in human skin. Immunohistochemical staining for BM-40 protein using the alkaline anti-alkaline phosphatase technique in adult human skin. Note (*a*) the pronounced staining in the papillary dermis and directly below the basement membrane zone (*scale bar*, 100 μm) and (*b*) the intercellular staining in suprabasal cell layers (→) in the epidermis shown at higher magnification (*scale bar*, 35 μm).

Because it is known that BM-40 protein synthesis is increased in human keratinocytes during sodium n-butyrate induced differentiation (Staiano-Coico *et al*, 1989; Ford *et al*, 1993), we asked the question whether the relatively low levels of BM-40 mRNA in keratinocytes might be due to the nondifferentiating conditions used for RNA isolation. To address this question keratinocytes were cultured in the presence or absence of 3 mM sodium n-butyrate for 24, 48, and 72 h. Northern blot analysis of RNA isolated from these cultures revealed a dramatic increase of BM-40 mRNA in the sodium n-butyrate treated keratinocytes already after 24 h when compared with untreated control cells (**Fig 2**). This result clearly indicates that the elevated protein level observed by Ford *et al* (1993) is due to an increase of the steady state BM-40 mRNA level.

BM-40 synthesis and deposition in cultured fibroblasts and keratinocytes In order to analyze BM-40 protein expression in keratinocytes and fibroblasts, cells were grown to confluency in normal growth medium and were subsequently cultivated for 48 h in serum-free medium. Western blot analysis of these conditioned media show that both cell types secrete BM-40 into the extracellular space (**Fig 3**). In contrast to melanoma cells that show specific cleavage of BM-40 (Ledda *et al*, 1997), only one band of about 42 kDa was detected in the supernatants of keratinocytes and fibroblasts.

Deposition of BM-40 was studied by indirect immunofluorescence with the BM-40 polyclonal anti-serum on unfixed keratinocytes and the keratinocyte-like cell line HaCaT (not shown) grown for 72 h on coverslides. Under these culture conditions keratinocytes only show signs of early differentiation. As seen in **Fig 4(*a*)**, the most prominent staining of BM-40 protein is detectable in the periphery of keratinocytes with suprabasal morphology, suggesting that BM-40 is either associated with the plasma membrane of these cells or secreted in the intercellular space. This is in contrast to the observation of Ford *et al* (1993) who found BM-40 deposition restricted to cornified envelopes. HeLa cells, as a negative control cell line lacking BM-40 expression (Hafner *et al*, 1994), were negative (**Fig 4*b***).

Localization of BM-40 mRNA and protein expression in human skin by *in situ* hybridization and immunohistochemistry To identify the cellular sources in the skin that are responsible for the high BM-40 mRNA level obtained by northern blot analysis, nonradioactive *in situ* hybridization using both digoxigenin labeled oligonucleotides (**Fig 5**) or anti-sense and sense strand riboprobes (results not shown) was performed. In the dermis expression was detected in fibroblasts throughout the whole compartment as well as in endothelial cells, smooth muscle cells, and glandular epithelial cells (**Fig 5*a*,*c***). In the epidermal compartment BM-40 mRNA expression is seen throughout the epidermis, being partly more pronounced in the basal cell layer (**Fig 5*a*,*b***).

Immunohistochemistry with the BM-40 anti-serum revealed staining in the whole dermal compartment, showing a clearly more pronounced signal directly below the basement membrane and in the papillary dermis (**Fig 6*a***). This finding coincides with the increased density of endothelial and fibroblast-like cells in the papillary dermis showing BM-40 mRNA expression. An increase in staining intensity could also be noted around vascular structures.

In the epidermis, staining for BM-40 was detected intercellularly most prominently in the suprabasal spinous and to a lesser degree in the basal and prickle cell layers (**Fig 6*b***). No staining was detected in the stratum corneum. This staining pattern clearly demonstrates that BM-40 expression *in vivo* is not restricted to the cornified cell layers of the epidermis.

DISCUSSION

Due to the origin of its purification, it had previously been assumed that the expression of BM-40 is mainly restricted to connective tissue and bone (Termine *et al*, 1981; Lankat-Buttgereit *et al*, 1988). As has been implicated from several *in vitro* studies (Howe *et al*, 1990; Wrana *et al*, 1991; Reed *et al*, 1993), BM-40 mRNA expression could be detected *in vivo* in fibroblasts throughout the dermis, in endothelial, smooth muscle and glandular epithelial cells. Increased deposition, as shown by immunohistochemistry, seems thereby to take place in areas supposedly characterized by an increased turnover of the surrounding connective tissue, i.e., papillary dermis, perivascular areas, and adnexal structures.

In the epidermis, as shown by *in situ* hybridization, the highest BM-40 mRNA expression was detected in cells of the basal layer. This indicates that the increase of BM-40 mRNA levels observed in sodium

n-butyrate differentiated keratinocytes might be due either to a higher expression rate or to stabilization of BM-40 mRNA mainly taking place in basal keratinocytes after induction of the differentiation process. In contrast, as shown by immunohistochemistry, there was no or only little protein detectable in this layer, indicating either that BM-40 mRNA is mainly translated in suprabasal cells or that the protein is readily deposited in a polarized fashion beneath the basal cell layer in the basement membrane zone or above in the extracellular space of the suprabasal cell layers. From our studies we cannot exclude that BM-40 is also localized intracellularly as has been recently shown by Porter *et al* (1995).

The role of BM-40 as an integral part of the epidermal intercellular space is unclear. But it is intriguing to speculate that BM-40, which has been classified as an "anti-adhesin" (Sage and Bornstein, 1991; Lane and Sage, 1994), might be involved in the migration process during epidermal differentiation by diminishing the contact of the basal keratinocytes to the underlying basement membrane and cell–cell contacts in the suprabasal layers. Whether BM-40, as a Ca^{2+} binding protein, is contributing to the Ca^{2+} gradient in the epidermis (Forslind, 1987) is unknown, and it remains to be seen whether the EF-hands in BM-40 fulfil a strictly structural role or whether they are used to transmit a Ca^{2+}-coupled signal as seen in the cytosolic homologs of the EF-hand family (for a review see Maurer *et al*, 1996).

Because BM-40 has been recently shown to be a substrate for transglutaminase catalyzed cross-linking in differentiating cartilage (Aeschlimann *et al*, 1995; Hohenadl *et al*, 1995), it is of interest to point out that the expression pattern in skin of an antibody generated to transglutaminase cross-links (Aeschlimann *et al*, 1995) parallels the expression pattern in the skin described herein for BM-40. This supports the possibility that BM-40 in the epidermis might be a target for transglutaminase catalyzed cross-linking, thereby altering biologic functions of this protein.

In summary, this study demonstrates that BM-40 is not only a component of the connective tissue of different organs but also part of epithelial structures such as the epidermis of adult human skin. Because BM-40 is detected in different epithelial tumors (Porter *et al*, 1995; Bellahcene and Castronovo, 1995), an understanding of the precise function of this protein in epithelial differentiation would also provide insight into the role of BM-40 in processes underlying tumor growth and invasion.

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