Basement Membrane Zone Remodeling During Appendageal Development in Human Fetal Skin. The Absence of Type VII Collagen is Associated with Gelatinase-A (MMP2) Activity

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Epithelial cell adhesion, migration, and differentiation are controlled by interactions at the basement membrane zone (BMZ). Type VII collagen is the major collagenous component of anchoring fibrils that are essential for the attachment of the epidermis to the dermis. Gelatinase A (MMP-2) is believed to be necessary for the degradation of type VII collagen. In this study we have examined the *in vivo* distribution of type VII collagen and gelatinase A (Gel A) in the developing human epidermis and its appendages. At 13–15 wk of gestation a marked decrease in type VII collagen immunoreactivity was seen in the BMZ surrounding invading appendageal buds; however, type VII collagen mRNA was strongly expressed in the budding epidermal keratinocytes adjacent to the BMZ. At these stages, Gel A-positive mesenchymal-like cells were found scattered throughout the stroma with numerous Gel A-containing cells in direct contact with the developing appendageal buds. *In situ* zymography was used to show Gel A-activity *in vivo*. Gel A-mediated lysis was present at the interface between the appendageal buds and the underlying BMZ. By 20–25 wk of gestational age, immunostaining for type VII collagen protein was absent from the BMZ surrounding the distal portion of invading appendageal epithelial cords of both hair follicles and sweat glands. In contrast, type VII collagen mRNA was present in the basal keratinocytes adjacent to the BMZ surrounding the distal portion of these invading appendageal epithelial cords. At these stages Gel A-positive cells were present in the stroma directly adjacent to the distal portion of developing appendageal cords that lacked type VII collagen. *In situ* zymography showed zones of Gel A-mediated stromal lysis at the distal portion of developing appendageal cords. Interestingly, no differences were seen in the distribution of type IV collagen in the BMZ of both budding and resting fetal epidermis. These observations suggest that the absence of type VII collagen protein correlates directly with the presence of Gel A-activity at the BMZ. Gel A appears to play a major role in appendageal development and contributes to remodeling of the BMZ during fetal skin morphogenesis.

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Type VII collagen is the major component of anchoring fibrils (AF). AF extend from the lamina densa of the basement membrane zone (BMZ) into the underlying reticular dermis and attach to anchoring plaques in the papillary dermis. Both AF and anchoring plaques form a complex network that entraps dermal extracellular matrix components (Lunstrum *et al*, 1986; Sakai *et al*, 1986; Keene *et al*, 1987; Burgeson *et al*, 1990). The importance of AF in BMZ stability is well established (Fine *et al*, 1984; Burgeson, 1993; Ljubimov *et al*, 1995; Craven *et al*, 1997) but the mechanisms regulating AF formation, type VII collagen deposition, and its degradation at the BMZ have not been fully elucidated. It has been shown that *in vitro* two proteases, human skin collagenase (MMP-1), and gelatinase A (MMP-2) can cleave type VII collagen (Seltzer *et al*, 1989; Galis *et al*, 1994; Kenney *et al*, 1994; Tournier *et al*, 1994; Yu *et al*, 1994). Gelatinase A (Gel A), however, is approximately 3000-fold more active against native type VII collagen than is interstitial collagenase (Seltzer *et al*, 1989).

In a previous study we showed that matrilysin (MMP-7) was expressed only by epidermal cells at the BMZ of invading appendageal cells (Karelina *et al*, 1994). Certain other members of the MMP family studied (interstitial collagenase, gelatinase B, stromelysin) were absent in both fetal epidermis and the extracellular stroma surrounding developing skin appendages. Here we show that type VII collagen is absent at the tips of developing appendageal buds, even though the basal keratinocytes of these buds continue to express type VII collagen mRNA. This loss of type VII collagen is associated with Gel-A-positive mesenchymal-like cells that are present at the BMZ surrounding the downward migrating appendageal epithelial cells as they invade the dermal stroma. *In situ* zymography shows that the areas of Gel A immunoreactivity colocalize with the regions of increased Gel A activity. These findings indicate that both a selective loss of type VII collagen and local Gel A-mediated stromal lysis may play an important role in localized extracellular matrix turnover required for fetal appendageal morphogenesis.

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MATERIALS AND METHODS

Fetal skin samples were obtained from spontaneous abortions at gestational ages from 6 to 28 wk. The following skin samples were studied: scalp (n = 28), palms (n = 25), and soles (n = 23). Normal skin samples (n = 5) were obtained from healthy volunteers after informed consent.

Immunohistochemical staining Specimens were embedded in "Tissue-Tek" O.C.T.4583 compound and stored at –70°C. Serial cryostat sections (5–7 μm in thickness) were placed on glass slides, air-dried, and fixed with 3.5% formaldehyde in Dulbecco's modified Eagle's phosphate-buffered saline (PBS) for 4 min, washed, and incubated with the primary antihuman type IV collagen antibody (Chemicon International, CA). The monoclonal antibody for type VII collagen (clone 4D2) was obtained from mice following immunization with a Triton insoluble fraction extracted from human epidermis (immunization and fusion were performed as described by Ljubimov *et al*, 1986). Clone 4D2 was selected on the basis of immunofluorescent staining of the BMZ of adult human skin. Purified pepsin-treated type VII collagen was subjected to SDS-PAGE. After transfer to PVDF paper, the gels were incubated with either polyclonal rabbit antibody against type VII collagen, or mouse monoclonal antibody clone 4D2, followed by the corresponding alkaline-phosphatase (AP)-conjugated secondary antibody. The specificity of this antibody was confirmed by western blot analysis (**Fig 1**). Type CVII collagen and polyclonal antitype VII collagen antibody were the kind gift of Dr. Robert Burgeson, Cutaneous Biology Research Center, MGH/Howard.

Both immunofluorescent and peroxidase-antiperoxidase (PAP) staining were used to detect type VII collagen in tissue sections. Double staining was performed using a tetramethylrhodamine B isothiocyanate (TRITC)-conjugated antimouse antibody and double exposure was carried out using Nikon filters BA520 for FITC and BA590 for Rhodamine B.

***In situ* zymography** To detect Gel A activity in tissue sections the method previously described by Galis *et al* (1994) was used. Glass slides with frozen tissue sections were dipped into a gelatin-containing photographic emulsion (NTB2, Eastman Kodak, Rochester, NY). Slides were incubated overnight in humidified chambers. Subsequently, the emulsion covered glass slides were dried, processed by photographic development, and examined microscopically using transmitted light. The background is black due to exposure of the emulsion to light. Lysis of gelatin in the emulsion produces transparent zones on the slides.

Zymogram analysis for Gel A Microdissected samples of human fetal skin (13–15 wk of gestation) were dissolved in six volumes of Laemmli's gel electrophoresis sample buffer (2% SDS, 40% glycerol, 12.5 mM TRIS, pH 7.5) at 370°C, incubated with DNAase, heated in boiling water, and centrifuged at 10,000 × *g* for 15 min and loaded on 8.75% gelatin gel.

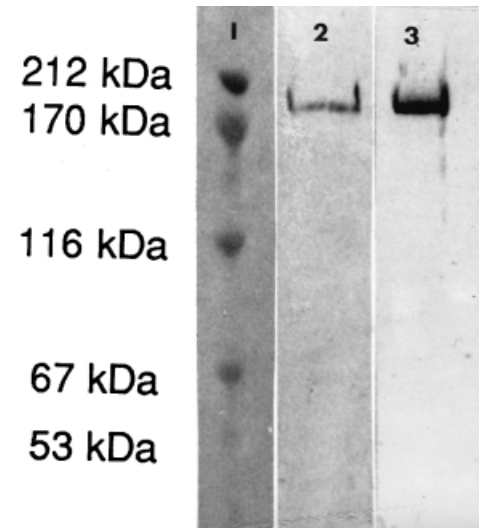

Figure 1. Western blot analysis of purified pepsin-treated type VII collagen incubated with antitype VII collagen antibody followed by AP-conjugated secondary antibody. See *Materials and Methods*. *Lane 1*, MW standards; *lane 2*, Polyclonal antitype VII collagen antibody; *lane 3*, monoclonal antitype VII collagen antibody (clone 4D2). Both antibodies recognized type VII collagen as a single band.

***In situ* hybridization**

Preparation of digoxigenin (Dig)-labeled mRNA probes The type VII collagen cDNA probe was made from a 728 bp Pvu II fragment of human K131 type VII collagen cDNA. Both sense and antisense mRNA probes were obtained using T7 and T3 RNA polymerases, respectively (a gift from Dr. W. Parks, Washington University School of Medicine). Digoxigenin-labeled antisense and sense RNA were synthesized using *in vitro* transcription kit (Boehringer, Mannheim, Germany). The size of the Dig-labeled RNA probes was checked on a formaldehyde gel. After ethanol precipitation the riboprobes were resuspended at 10 μg per μl in a buffer (pH 7.5) consisting of 10 mM Tris HCl and 1 mM ethylenediamine tetraacetic acid.

Hybridization procedure Samples for *in situ* hybridization were embedded in "Tissue-Tek" O.C.T.4583 compound, snap frozen in liquid nitrogen, and stored at –70°C until used. Paraformaldehyde-fixed cryostat sections (5–7 μm in thickness) were incubated in 20 μl of hybridization solution containing 50% deionized formamide; 0.3 M NaCl; 20 mM TrisHCl (pH 7.4); 5 mM EDTA; 10 mM $NaH_2PO_4xH_20$ (pH 8.0); 10% dextran sulfate; 1 × Denhardt's; and 0.5 mg per ml total yeast RNA. Hybridization was carried out overnight at 50°C in a humidified chamber after addition of the diluted (to a final concentration of 0.5 μg per μl) and heat-denatured probes. After the hybridization, nonhybridized probes were removed by washing procedures with 4 × SSC followed by washing with 2 × SSC and 0.1 × SSC. The tissue sections were incubated with a 1:2000 dilution of an AP-labeled anti-Dig antibody (Boehringer) overnight. Staining of the tissue sections was performed using a freshly prepared color-substrate solution containing 200 μl NBT/BCIP (Boehringer).

RESULTS

At 6–8 wk of gestation, when fetal epidermis begins to stratify from a two to a three-layered epithelium, type VII collagen protein was first seen as a weakly staining band at the BMZ (**Fig 2*A***). At this gestational stage a strong signal for type VII collagen mRNA was localized to the basal cells along the BMZ (**Fig 2*B***). Prior to this time (5 wk of gestational age) although immunostaining for type VII collagen was absent at the BMZ, a linear type IV collagen-positive fluorescent band was observed along the BMZ of the developing epidermis (not shown). At these early stages of cutaneous development (between 6 and 8 wk of gestation), Gel A-positive mesenchymal-like cells were scattered throughout the stroma (**Fig 2*C***). *In situ* zymography was used to localize Gel A-catalytic activity in the developing skin and showed that at 6–8 wk of gestation there was no specific enzyme-mediated lysis in the underlying dermis (not shown).

By 10 wk of gestation, type VII collagen was present as an intensely staining linear fluorescent band along the BMZ (**Fig 2*D***) and a prominent signal for type VII collagen mRNA was concentrated in the basal keratinocytes along the dermo–epidermal junction (**Fig 2*E***). Weak to moderate Gel A-activity was present in the upper stromal layers at this stage of development.

At the time of appendageal bud formation (between 13 and 15 wk of gestational age), staining for type VII collagen was present at the BMZ of the interappendageal basal cells, but type VII collagen-immunoreactivity was completely absent from the BMZ surrounding the developing appendageal buds (**Fig 2*F***). Type IV collagen (and laminin, data not shown), however, remained present along the entire BMZ of both the interappendageal regions, and the budding hair follicles and sweat glands (**Fig 2*G***). Importantly, a strong signal for type VII collagen mRNA expression was found in the appendageal buds, where staining for type VII collagen was absent (**Fig 2*H***, ***I*** and **Fig 3*A***). During this developmental period Gel A-positive stromal cells became more abundant and were localized in the stroma adjacent to the BMZ of the developing appendages (**Fig 2*J*** and **Fig 3*B***). Scattered Gel-A-positive mesenchymal cells were also seen through-

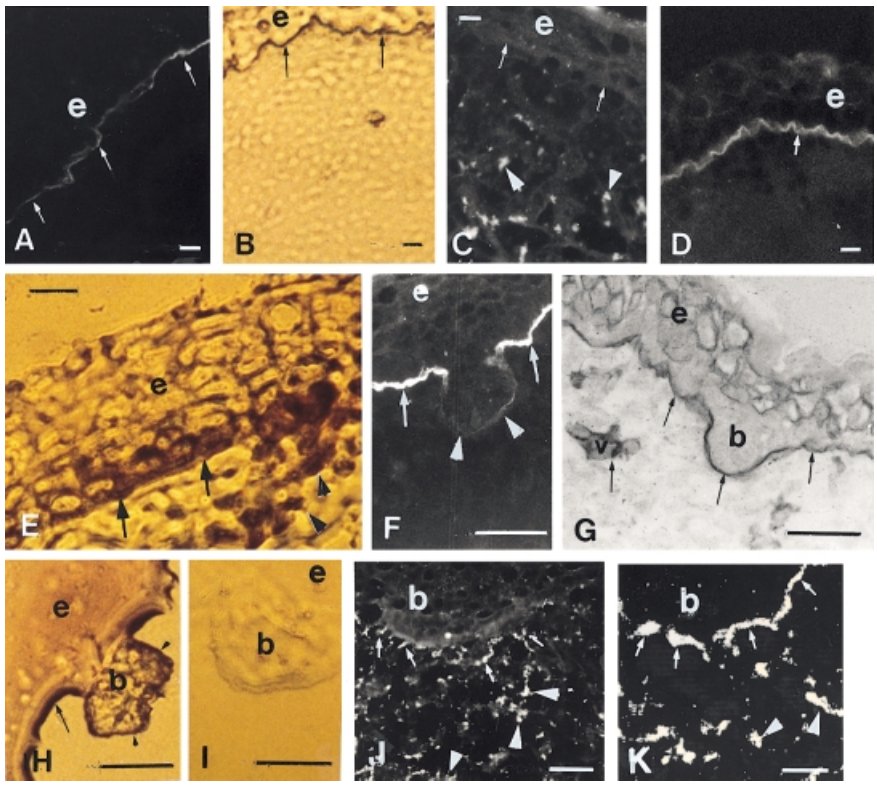

Figure 2. Distribution of type VII collagen and Gel A in developing skin at 8–13 wk of gestation. (*A–C*) Fetal skin at 8 wk of gestation. Immunofluorescent staining for type VII collagen (*A*); weak staining is seen along the BMZ (*arrows*) of the developing epidermis (e). (*B*) Type VII collagen mRNA expression (*arrows*, dark brown color) is restricted to the basal keratinocytes lining the BMZ; (*C*) Gel A-positive cells are scattered throughout the developing stroma (*arrowheads*). No immunostaining for Gel A is present along the BMZ (*arrows*). *Scale bar*: 5 μm. (*D*) Fetal skin at 10 wk of gestation. Immunofluorescent staining for type VII collagen. An intensely staining linear fluorescent band is seen along the BMZ (*arrow*); e, epidermis. *Scale bar*: 5 μm. (*E*) Expression of type VII collagen mRNA in fetal skin at 12 wk of gestation. A strong signal is seen in the basal keratinocytes adjacent to the BMZ (*arrows*, dark brown color). Some fibroblast-like stromal cells close to the BMZ also show a positive signal (*arrowheads*). *Scale bar*: 25 μm. (*F*) Fetal skin at 13 wk of gestation. Immunofluorescent staining for type VII collagen shows that this protein extends along the BMZ (*arrows*) of the interappendageal basal cells, but is completely absent (*arrowheads*) from the BMZ surrounding a developing appendageal bud. *Scale bar*: 20 μm. (*G*) Immunoperoxidase-antiperoxidase-staining for type IV collagen in fetal skin at 13 wk of gestation. Type IV collagen is present as a linear band along the entire BMZ (*arrows*) of a developing appendageal bud (b) and dermal blood vessels (v). *Scale bar*: 20 μm. (*H*) Fetal skin at 13 wk of gestation. Expression of type VII collagen mRNA is restricted to the BMZ (*arrow*) and a strong signal is seen predominantly in the basal keratinocytes of the appendageal buds (*arrowheads*; b, bud). *Scale bar*: 20 μm. (*I*) Control section of (*H*). No specific signal is present in the developing appendageal bud (b) and epidermis (e). *Scale bar*: 20 μm. (*J*) Immunofluorescent staining for Gel A at 13 wk of gestation. Gel A-positive cells are present in the upper stromal layers (*arrowheads*) and are localized (*arrows*) at the BMZ of a developing appendageal bud (b). *Scale bar*: 25 μm. (*K*) *In situ* zymography showing Gel A activity at 13 wk of gestation. Bright zones of lysis by Gel A are present in the stroma (*arrowheads*) and along the developing appendageal bud (*arrows*); b, bud. *Scale bar*: 25 μm.

out the stromal layers (**Fig 2*J***). Beginning with these gestational stages, *in situ* zymography showed an increase in Gel A-activity in the stroma surrounding the developing appendageal buds (**Fig 2*K***) that correlated directly with the absence of type VII collagen (**Fig 2*F***). The presence of active enzyme in fetal epidermis was confirmed by showing that Gel A activity was present in tissue extracts (**Fig 4**) at the same gestational stages.

As the appendageal buds begin to invade the extracellular stroma (15–18 wk of gestation), type VII collagen, although present along the appendageal BMZ, remained absent at the distal portion of the invading hair follicles and sweat glands (**Fig 3*C*, *D***). In contrast, the expression of type VII collagen mRNA was present in the invading keratinocytes of the developing appendageal buds (**Fig 3*E***). A type IV collagen-positive fluorescent band, however, was found along the BMZ of the invading appendages (**Fig 3*F***). Gel A-containing mesenchymal cells continued to be concentrated in the stroma adjacent to the distal portion of the invading appendageal buds that lacked type VII collagen (**Fig 3*G*, *H***). *In situ* zymography showed zones of prominent Gel A-mediated activity in the stroma surrounding the developing appendageal buds of both hair follicle and sweat gland appendages (**Fig 3*B*, *J***).

By 20–28 wk of gestation immunostaining for type VII collagen was more intense than that seen in early developmental stages and was present along the full extent of the BMZ (not shown). At these stages Gel A-positive mesenchymal cells were less abundant at the BMZ but remained in the stroma surrounding the distal portion of developing hair follicles (**Fig 3*I***) and in the dermal papilla (**Fig 3*J***).

In adult human skin type VII collagen was present in the BMZ, the hair follicles, and sweat gland ducts. The distal portions of both types of appendages continued to stain weakly for type VII collagen. It was no longer possible to detect Gel A either by *in situ* zymography or by immunostaining in the mature stroma of normal adult skin.

DISCUSSION

Type VII collagen is an integral component of anchoring fibrils. AF secure the epidermis to the dermis and help to maintain cell–cell and cell–matrix interactions (Konig and Bruckner-Tuderman, 1991, 1992; Gammon *et al*, 1992; Ryynanen *et al*, 1992; Agha-Mir-Salim *et al*, 1993; Marinkovich *et al*, 1993; Chen *et al*, 1994; Mauviel *et al*, 1994). Abnormalities of type VII collagen, for example, have been shown to be the cause of skin fragility and blister formation in recessive dystrophic epidermal bullosa (Shimizu *et al*, 1990; McGrath *et al*, 1992; Phillips *et al*, 1992; Fine *et al*, 1993; Hovnanian *et al*, 1994; Kalinke *et al*, 1994; Konig *et al*, 1994a, b).

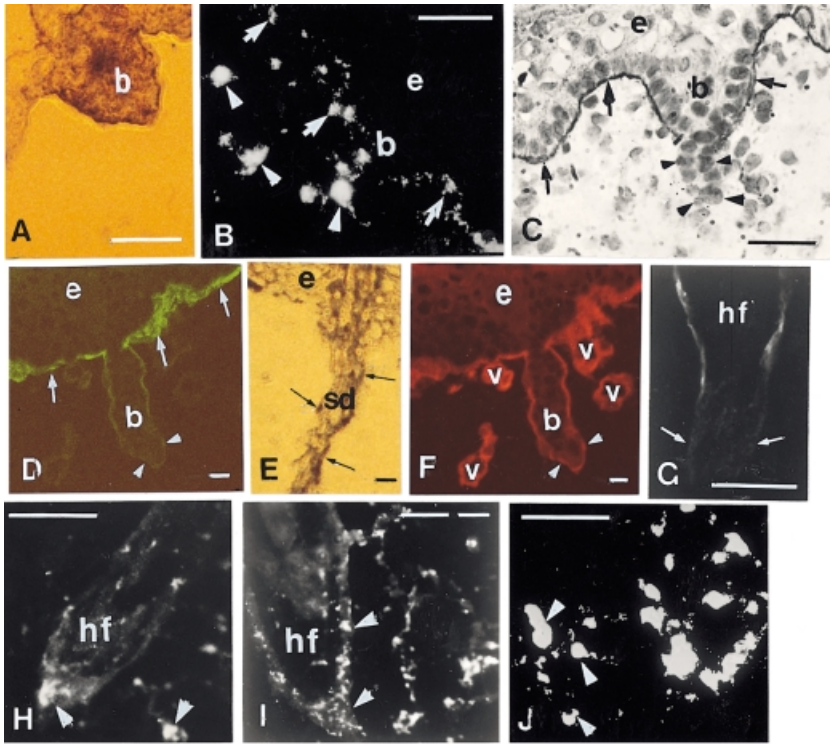

Figure 3. Distribution of Gel A and type VII collagen in developing skin at late (15–28 wk) gestational stages. (*A*) Fetal skin at 15 wk of gestation. *In situ* hybridization for type VII collagen mRNA shows a strong intracellular signal in the keratinocytes of the entire appendageal bud (b, bud, brown color). *Scale bar*: 20 μm. (*B*) *In situ* zymography of serial section from the tissue sample shown in (*A*). Gel A activity is shown by the bright zones of lysis distributed along the BMZ (*arrows*) and in the stroma (*arrowheads*) adjacent to the developing appendageal bud (b, bud; e, epidermis). *Scale bar*: 20 μm. (*C*) Fetal skin at 15 wk of gestation. Immunoperoxidase-antiperoxidase staining for type VII collagen shows that the protein remains absent (*arrowheads*) from the distal portion of the invading appendageal bud (b) but is present along the entire interappendageal BMZ (*arrows*); e, epidermis. *Scale bar*: 20 μm. (*D*) Fetal skin at 18 wk of gestation. Immunofluorescent staining for type VII collagen. Type VII collagen remains absent at the distal portion of a developing appendageal bud (b, *arrowheads*) but is present along the interappendageal BMZ (*arrows*); e, epidermis. *Scale bar*: 10 μm. (*E*) *In situ* hybridization for type VII collagen mRNA in a developing appendageal sweat gland duct (sd). A strong signal is present in the keratinocytes of the invading appendageal sweat gland (*arrows*, dark brown color); e, epidermis. *Scale bar*: 10 μm. (*F*) In contrast to (*D*), type IV collagen completely surrounds the developing appendageal bud (b, *arrows*) and blood vessels (v); e, epidermis. *Scale bar*: 10 μm. (*G*) Fetal skin at 18 wk of gestation. Type VII collagen continues to be absent (*arrows*) from the BMZ at the distal portion of an invading hair follicle (hf). *Scale bar*: 25 μm. (*H*) Serial section from the same tissue sample shown in (*G*) immunostained for Gel A. Gel A-positive cells (*arrowheads*) are present in the stroma and along the invading portion of the developing hair follicle (hf). *Scale bar*: 25 μm. (*I*) immunofluorescent staining for Gel A at 25 wk of gestation. Gel A-positive cells (*arrowheads*) are distributed in close association to the BMZ in the invading hair follicle (hf). *Scale bar*: 25 μm. (*J*) *In situ* zymography of a serial section from the sample shown in (*I*) but at higher magnification. Gel A activity (*arrowheads*) colocalizes with the Gel A-positive immunostaining cells at the BMZ of the developing hair follicle seen in (*I*). *Scale bar*: 25 μm.

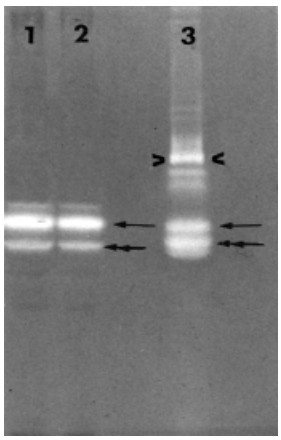

Figure 4. Zymography showing the presence of Gel A activity in tissue extracts. Microdissected samples of human fetal skin were prepared as described in *Materials and Methods*. *Lane 1*, 5 μl of sample lysate; *lane 2*, 3 μl of sample lysate; *lane 3*, pure Gel A showing the proenzyme (72 kDa, *arrow*) and the active (62 kDa, *double arrow*) enzyme species. The *arrowheads* are pure Gel B (92 kDa, MMP-9).

Epithelial–mesenchymal interactions established during early fetal development are necessary for efficient deposition of type VII collagen (Bruckner-Tuderman *et al*, 1987; Leigh *et al*, 1987; Smith *et al*, 1988; Ryynanen *et al*, 1992). When examining the expression and distribution of type VII collagen during skin morphogenesis, we found that at early gestational stages of development (5–6 wk) the BMZ showed a low level immunostaining for type VII collagen and a weak signal for type VII collagen mRNA; both became more intense with increasing gestational age. Of particular interest was the finding that during appendageal bud formation (13–15 wk of gestational age) there was a loss of type VII collagen from the BMZ surrounding the invading appendageal buds. This occurred in the presence of continued type VII collagen mRNA expression in the basal epithelial cells of these developing appendages. These data clearly show that there are specific temporal and regional differences in the distribution of type VII collagen along the BMZ during specific stages of appendageal development. In contrast, in the mature fetal epidermis (at 23–30 wk of gestation), type VII collagen was evenly distributed along the entire BMZ including the skin appendages.

Of particular importance was the finding that the disappearance of type VII collagen at the site of appendageal invasion into the dermis was associated with an increased number of Gel A-producing stromal cells. The Gel A producing stromal cells were present at the interface between the appendageal buds and

underlying extracellular matrix. These data are consistent with the *in situ* zymographic localization of Gel A-activity. Significant Gel A-mediated lysis was localized to the mesenchymal-like cells underlying the BMZ in fetal dermis at the sites of type VII collagen loss. Based on this observation, we propose that the absence of type VII collagen with the attendant loss of AF may facilitate the invasion of budding appendageal epithelial cells into the dermis.

These results support the hypothesis that epithelial and stromal cells cooperate to achieve matrix degradation and promote tissue invasion during normal appendageal development (Ryynanen *et al*, 1992; Kalinke *et al*, 1994; Basbaum and Werb, 1996; Craven *et al*, 1997). Our *in situ* hybridization findings show that there is type VII collagen mRNA present and presumably synthesis of this protein in the basal cells of the appendageal buds during their morphogenesis. Thus, the loss of type VII collagen at the site of invading appendageal buds may be the result of enzymatic degradation by Gel A of type VII collagen during this invasive process. It has been shown that *in vitro*, Gel A is capable of rapidly degrading native type VII collagen (Seltzer *et al*, 1989). Our data are supported by the observation that in fetal rat, glandular bud formation during tracheal development is associated with the presence of Gel A in both the surrounding stroma and the invading glandular epithelium (Tournier *et al*, 1994). This suggests that Gel A mediates the transition from a noninvasive epithelial cell to an invasive cell by degrading specific extracellular matrix components.

In our previous studies to define the role of MMP in appendageal development in fetal skin (Karelina *et al*, 1994), we found that matrilysin (MMP-7) was concentrated in epithelial cells at the distal portion of the invading follicular and sweat gland appendages. This enzyme, expressed by invading epithelial cells, may act in concert with Gel A to degrade type VII collagen and perhaps other BMZ components. The results of this study show that the production of Gel A by mesenchymal-like cells in the developing dermis plays a significant role in appendageal invasion and contributes to the proteolytic activity required for rapid, localized, extracellular matrix turnover.

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