Type VII Collagen Gene Expression by Human Skin Fibroblasts and Keratinocytes in Culture: Influence of Donor Age and Cytokine Responses

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Type VII collagen is the predominant, if not the exclusive, component of the anchoring fibrils. In this study, we have examined the expression of the type VII collagen gene in human skin fibroblasts and keratinocytes in culture by Northern analyses and immunocytochemistry. Type VII collagen gene expression was greatly enhanced in all cell strains studied after stimulation by transforming growth factor-β (TGF-β). However, no definitive correlation between the donor age and the magnitude of TGF-β response could be made. In contrast, the basal expression of the type VII collagen gene was shown to decrease in an age-dependent manner in fibroblasts. The pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were shown to elevate type VII collagen mRNA levels in a dose-dependent manner. This response was inversely related to the donor age of the cell cultures. The attenuated response of cells from older individuals to TNF-α and IL-1β was specific for type VII collagen gene expression, because, in the same experiments, collagenase gene expression was strongly elevated by the two cytokines. Our data suggest that type VII collagen gene expression is subject to modulation by the cytokine network, which may play a role in controlling anchoring fibril assembly in normal skin and in pathologic conditions characterized by altered deposition of type VII collagen. *Key words: collagen gene expression/donor age/cytokine responses. J Invest Dermatol 102:205–209, 1994*

Collagens comprise a family of closely related yet genetically distinct proteins, and at present 18 collagens (types I-XVIII) have been characterized either at the protein and/or nucleic acid level [1–4]. Among these 18 collagens, at least 11 of them have been identified in the skin [5]. Some of the collagens present in the skin, such as type VII collagen, demonstrate a topographically restricted localization. Specifically, type VII collagen is the predominant, if not the exclusive, component of the anchoring fibrils, attachment structures present in the basement membrane zone of stratified squamous epithelia, i.e., the skin, the mucous membranes, and the cornea of the eye [6,7].

Previous studies utilizing immunologic techniques have suggested that both dermal fibroblasts and epidermal keratinocytes in culture synthesize type VII collagen [8–10]. More recently, cloning of human type VII collagen cDNAs has allowed demonstration of the corresponding gene expression at the mRNA level [11,12]. Northern analyses and immunocytochemistry have demonstrated its upregulation by transforming growth factor-β (TGF-β) [13,14].

The presence of anchoring fibrils is apparently critical for the stability of the cutaneous basement membrane zone, because structural abnormalities, paucity, or even absence of anchoring fibrils manifest clinically as the dystrophic (scarring) forms of epidermolysis bullosa (EB), characterized by cutaneous fragility and tendency to sub-basal lamina blister formation [15–17].

A previous study has suggested that topical application of all-*trans*-retinoic acid onto photodamaged skin results in a significant increase in the number of anchoring fibrils, which were apparently scarce in the untreated skin [18]. Furthermore, recent studies have suggested that the effects of all-*trans*-retinoic acid upon skin could be mediated, at least in part, by increased expression of TGF-β [19,20]. In this study, we have examined the response of fibroblasts and keratinocytes from individuals of varying ages to TGF-β, as well as to tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), members of the cytokine network, which have been previously shown to modulate the expression of the extracellular matrix genes [21].

MATERIALS AND METHODS

Cell Cultures Human neonatal epidermal keratinocytes from foreskin or adult keratinocytes from sun-protected mammoplasty skin specimens were obtained from Clonetics Corp. (San Diego, CA), and the cultures were maintained in serum-free, low-calcium (0.15 mM) keratinocyte growth medium supplemented with epidermal growth factor, hydrocortisone, insulin, and bovine pituitary extract (KGM; Clonetics). Human skin fibroblast cultures were established from mammoplasty skin specimens obtained from sun-protected areas from individuals of varying ages. Fibroblast cultures from fetal skin were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The fibroblast cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 50 μg/ml streptomycin, 200 U/ml penicillin-G, and 10% fetal bovine serum (FBS). One hour prior to the addition of growth factors, the fibroblast cultures were rinsed with DMEM and placed in DMEM containing 1% FBS.

TGF-β1, purified from bovine bone, and human recombinant TGF-β2 were kindly provided by Dr. David R. Olsen, Celtrix Laboratories, Santa

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Clara, CA. Human recombinant IL-1β and TNF-α were purchased from Boehringer Mannheim, Indianapolis, IN.

Indirect Immunofluorescence Cell cultures were established on chamber slides (Nunc Co., Naperville, IL), stained with a monoclonal anti-type VII collagen antibody L_3D [22] (kindly provided by Dr. R.A. Briggaman, University of North Carolina, Chapel Hill), and processed in parallel, as described previously [10,13].

Northern Analysis Total RNA was isolated either by a single-step procedure [23] or by $CsCl_2$ density gradient centrifugation [24]. Northern filters were prepared as described elsewhere [11,13], and hybridized with a 1.9-kb human type VII collagen cDNA K-131 [11]. The same filters were rehybridized with a human GAPDH cDNA (American Type Culture Collection, Rockville, MD), as described previously [13].

RESULTS

TGF-β Upregulates Type VII Collagen Synthesis in Cultured Fibroblasts from Individuals of Varying Ages To examine the effect of age on type VII collagen gene expression, fibroblast cultures were derived from four subjects varying from 8 weeks fetal gestation to 75 years of age. All cultures were examined at passages 3–5 under identical culture conditions. A monoclonal antibody (L_3D) recognizing an epitope within the NC-1 domain was utilized for indirect immunofluorescence, and the intensity of the immunosignal was taken as a semi-quantitative indicator of the level of type VII collagen production (Fig 1). Immunostaining of control cultures incubated in medium containing 1% FBS demonstrated a low, yet clearly detectable, level of type VII collagen epitopes in cultures from all ages (Fig 1*A–D*). Because of the low level of the signal, no precise assessment of the level of type VII collagen gene expression in control fibroblast cultures as a function of the age of the skin donor could be made.

To examine the effect of TGF-β1 on type VII collagen synthesis, parallel cultures were incubated with TGF-β1 (10 ng/ml). This concentration was previously shown to result in maximal enhancement of type VII collagen synthesis in cultured keratinocytes [13]. The addition of TGF-β1 to the culture medium markedly enhanced type VII collagen synthesis in all four donor cultures, as visualized by indirect immunofluorescence (Fig 1*E–H*) after 48 h of incubation. In this experiment, the cell cultures established from a 75-year-old individual appeared to be somewhat less responsive to TGF-β1, as judged by the intensity of the immunofluorescence staining (Fig 1*H*). The cells established from the skin of an 8-week-old fetus also appeared to be less responsive to TGF-β than the cells from the young adults (Fig 1*E* versus 1*F*). This finding may relate to the observation that type VII collagen gene expression is initiated relatively late during fetal skin development, and anchoring fibrils can not be detected ultrastructurally until about the twelfth week of gestation [25]. Thus, the cells from a fetus of 8 weeks gestation may not have the capacity to fully respond to TGF-β1. This interpretation was further supported by the observation that fibroblasts from a fetus of 20 weeks gestation were more responsive to TGF-β at the mRNA level (see below). Nevertheless, fibroblasts derived from the skin of individuals of all ages examined in this experiment were clearly responsive to TGF-β1 with respect to type VII collagen synthesis (Fig 1). Similar enhancement of type VII collagen synthesis was also noted in the presence of TGF-β2 (not shown).

Effects of TGF-β on Type VII Collagen mRNA Levels The effects of donor age and response to TGF-β were also examined at the mRNA level. As illustrated by Fig 2, Northern analyses with a human type VII collagen cDNA of total RNA isolated from three cell cultures established from individuals of 23, 41, or 84 years of age demonstrated an age-dependent decrease in the basal level of type VII collagen gene expression. Specifically, the levels of type VII collagen mRNAs, after correction for the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same RNA preparations, in cultures from a 23-year-old donor were approximately twice that noted in the 84-year-old donor's culture (Fig 2). An intermediate level was noted in cultures established from the 41-year-old individual.

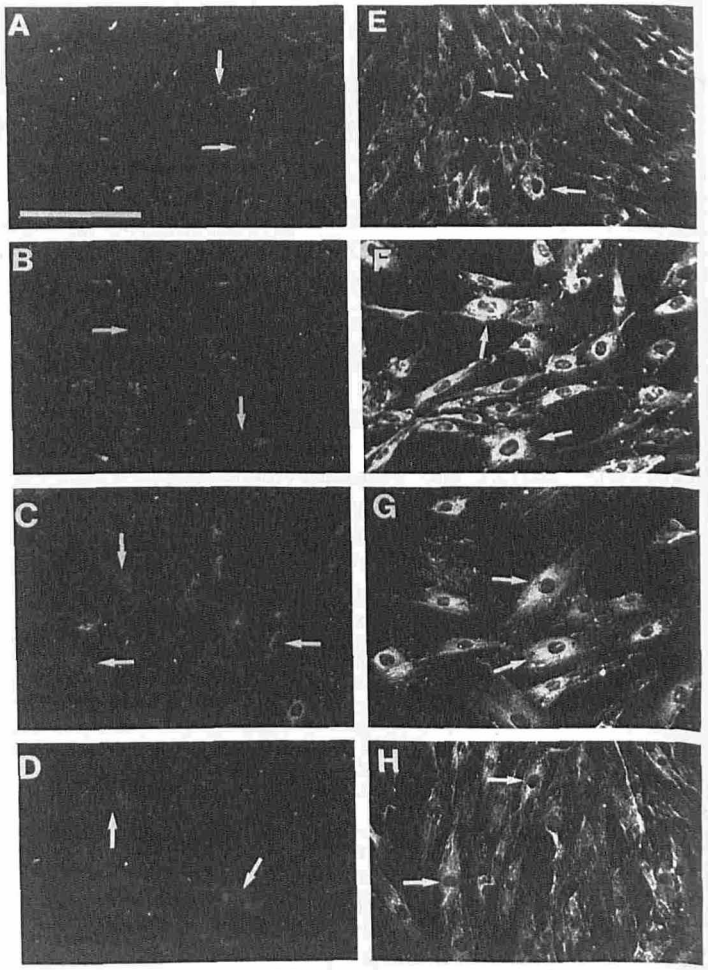

Figure 1. Stimulation of type VII collagen synthesis by TGF-β1 in fibroblast cultures established from individuals of varying ages. The cell cultures were established from fetal (8-week gestation; *A* and *E*), 21-year-old (*B* and *F*), 55-year-old (*C* and *G*), or 75-year-old (*D* and *H*) individuals. The fibroblast cultures were incubated in parallel without *(A–D)* or with *(E–H)* TGF-β1 (10 ng/ml) for 48 h. The type VII collagen epitopes were visualized by immunostaining with a monoclonal antibody, L_3D, as described in *Materials and Methods.* Immunostaining is identified by *arrows. Bar,* 200 μm.

To examine the response of the fibroblast cultures to TGF-β at the mRNA level, the cultures shown in Fig 2 were also incubated in parallel with TGF-β1 (10 ng/ml). The results indicated that all three cell cultures responded to TGF-β, and the extent of stimulation varied from 1.6 to 4.8 times (Table I). Similar enhancement of type VII collagen gene expression, as determined at the mRNA level, was observed in five additional cell strains, and the stimulation by TGF-β varied from 2.2 to 10.4 times (Table I). Thus, these experiments, which examined a total of 12 fibroblast cell strains established from individuals varying from 8 weeks fetal gestation to 84 years of age, collectively indicated that cultured skin fibroblasts from normal individuals are responsive to TGF-β with respect to type VII collagen gene expression.

TGF-β Enhances the Synthesis of Type VII Collagen in Epidermal Keratinocytes To examine the possible age variation and the enhancement of type VII collagen synthesis by TGF-β in cultured keratinocytes, indirect immunofluorescence analyses were performed on neonatal and adult keratinocytes incubated for 48 h with or without TGF-β1 (10 ng/ml). Clearly detectable immunosignal was observed in both types of keratinocyte cultures (not shown). Furthermore, a markedly enhanced immunosignal in TGF-β1–treated cultures was noted, and the enhancement ap-

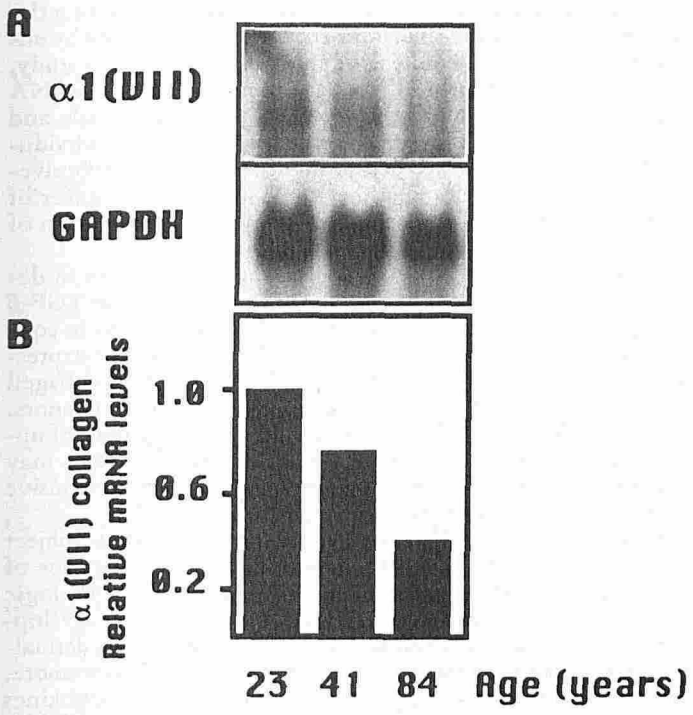

Figure 2. Effect of age of the donors of tissue specimens used to establish fibroblast cultures on type VII collagen gene expression. Fibroblast cultures were established from individuals of 23, 41, or 84 years of age, total RNA was isolated, and Northern hybridizations were performed with α1(VII) collagen and GAPDH cDNAs *(A)*. The relative levels of the α1(VII) collagen mRNAs as a function of age, as determined by scanning densitometry of the mRNAs, after correction for the levels of the GAPDH mRNA in the same preparation, are indicated in *B*.

peared to be equally strong in neonatal and adult keratinocyte cultures. The enhancement of type VII collagen gene expression by TGF-β in these cultures was also demonstrated at the mRNA level, in a similar manner to that shown previously [13].

Effects of IL-1β on Type VII Collagen Gene Expression in Dermal Fibroblasts To examine the effects of other growth factors, cultured fibroblasts were first incubated in the presence of IL-1β, in concentrations varying from 0 to 10 U/ml. Incubation of cells from a 19-year-old individual demonstrated a marked, up to twelvefold, enhancement of type VII collagen gene expression in a

Table I. TGF-β Stimulation of Type VII Collagen Gene Expression[a]

Fibroblast Donor	*Fold Stimulation* of α1(VII) mRNA by TGF-β
20-week-old fetus	10.4
Newborn 1	2.5
Newborn 2	3.2
Newborn 3	4.9
23 year old[b]	1.6
41 year old[b]	2.0
73 year old	2.2
84 year old[b]	4.8

[a] Cultured fibroblasts were incubated for 24 h with or without TGF-β (10 ng/ml). At the end of incubations, total RNA was extracted and analyzed by Northern hybridization for type VII collagen gene expression. Filters were re-hybridized with a GAPDH cDNA as a control. Autoradiograms were analyzed by scanning densitometry, and the extent of stimulation of type VII collagen mRNA by TGF-β was calculated after correction for GAPDH mRNA levels in the same RNA preparations.

[b] These donors are the same as in Fig 2.

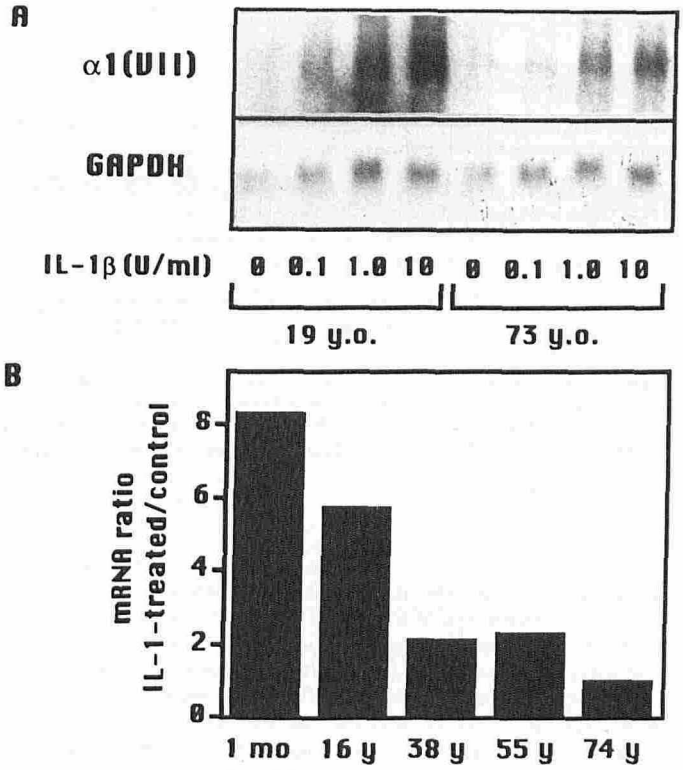

Figure 3. Effects of IL-1β on type VII collagen gene expression in dermal fibroblast cultures established from the skin of individuals of varying ages. *A)* Fibroblast cultures established either from a 19-year-old or a 73-year-old individual were incubated with IL-1β in varying concentrations (0–10 U/ml) for 24 h. Total RNA was isolated and the α1(VII) collagen and GAPDH mRNAs were detected by Northern hybridizations with the corresponding cDNAs. *B)* A set of fibroblast cultures established from the skin of individuals varying from 1 month to 74 years of age were incubated without or with IL-1β (1 U/ml) for 24 h, and the enhancement of α1(VII) collagen mRNA levels was determined by scanning densitometry of Northern blots. The relative values, after correction by GAPDH levels in the corresponding mRNA samples, are depicted.

dose-dependent manner (Fig 3*A*). Similar, although less pronounced, enhancement was noted in cultures established from a 73-year-old individual (Fig 3*A*). Further analysis of fibroblast cultures established from individuals varying from 1 month to 74 years of age demonstrated that the response to IL-1β diminished with advancing age of the donor (Fig 3*B*). Specifically, the enhancement of the α1(VII) collagen mRNA levels in fibroblast cultures from the 1-month-old donor incubated with IL-1β (1 U/ml) was approximately eightfold, whereas little enhancement was noted in the cultures from a 74-year-old donor (Fig 3*B*). Thus, human dermal fibroblasts in cultures established from younger donors demonstrate a clear enhancement of type VII collagen gene expression by IL-1β. However, this response was inversely related to the donor age of the cell cultures, and the eldest individuals' cells showed lesser, and somewhat variable, response. This variability may reflect the low level of expression in the elderly individuals.

Effects of TNF-α on Type VII Collagen Gene Expression in Dermal Fibroblasts To further extend the investigation of effects of the cytokine network on type VII collagen gene expression, fibroblast cultures established from 21- and 73-year-old donors were incubated with varying concentrations of TNF-α, a potent inhibitor of type I collagen gene expression [26–29]. Incubation of fibroblast cultures established from the 21-year-old donor with TNF-α resulted in clear enhancement of the type VII collagen gene expression, ~2.5-times enhancement being noted in the presence of 20 ng/ml of TNF-α (Fig 4). In contrast, the cell cultures established

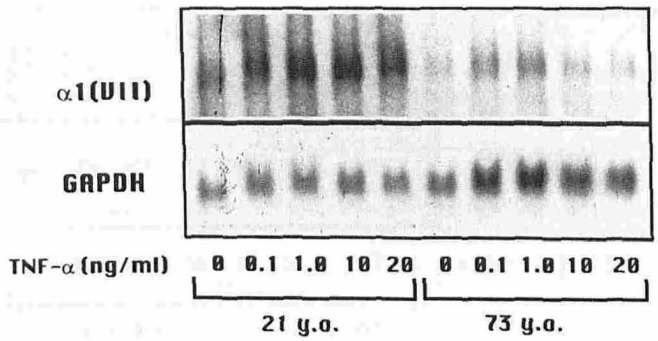

Figure 4. Effects of TNF-α on type VII collagen gene expression in fibroblast cultures established from skin of donors of 21 or 73 years of age. The fibroblast cultures were incubated with TNF-α in concentrations varying from 0 to 20 ng/ml. Northern hybridizations depict the presence of α1(VII) collagen and GAPDH mRNAs.

from the 73-year-old individual demonstrated essentially no change in the presence of TNF-α in concentrations up to 20 ng/ml, after correction for the densitometric values of the GAPDH mRNA levels in the same samples (Fig 4). It should be noted that the latter cells were clearly responsive to TNF-α with respect to collagenase gene expression, which was increased by up to 6.2 times in the presence of TNF-α (not shown). Thus, the cultured human skin fibroblasts appear to demonstrate a differential, age-dependent response to TNF-α.

DISCUSSION

The critical importance of anchoring fibrils in providing the integrity to the cutaneous basement membrane zone is attested to by the fact that abnormalities in the synthesis of type VII collagen or its assembly to anchoring fibrils result in a scarring blistering disease, the dystrophic form of EB [15–17]. In fact, we have recently demonstrated that some of the recessive dystrophic EB patients contain structural mutations in the α1(VII) collagen gene [30,31], whereas other studies have suggested deficient expression on the translational or post-translational level [32]. These observations have then suggested that upregulation of type VII collagen gene expression might be helpful in ameliorating or even reversing the blistering tendency in some cases with the dystrophic forms of EB [15].

Previous studies have indicated that TGF-β1 and TGF-β2, members of the pleiotropic TGF-β cytokine family, are capable of upregulating type VII collagen gene expression in cultured dermal fibroblasts and epidermal keratinocytes [13,14]. In this study, we have extended these observations to a variety of fibroblast cultures established from individuals varying from 8 weeks fetal gestation to 84 years of age. The studies on fibroblasts demonstrate that cultures established from any of the donors of any age studied respond significantly to TGF-β by upregulating their type VII collagen gene expression, as detected either by indirect immunofluorescence at the protein level or by Northern hybridizations at the mRNA level. However, no apparent difference in the degree of response to TGF-β could be noted between cultures established from individuals of varying ages. Recent indications in the literature have suggested that the number of anchoring fibrils may decrease with advancing age on the sun-exposed area of skin [18]. Thus, some age-associated manifestations in the skin, such as fragility and impaired epidermal wound healing, might be attributable to reduced anchoring fibrils and diminished type VII collagen synthesis in the elderly individuals.

In contrast to TGF-β, no information has been available on the effects of other cytokines on type VII collagen gene expression. In this study, two members of the cytokine network, IL-1β and TNF-α, were tested for their effects on type VII collagen gene expression in human skin fibroblast cultures. These two cytokines in general play a role in tissue remodeling and degradation by modulating the biosynthesis of the extracellular matrix macromolecules and by enhancing the synthesis of proteolytic enzymes [21,33]. In this study, IL-1β was shown to markedly elevate type VII collagen mRNA levels in fibroblast cultures established from young individuals, and the response diminished with advancing age of the donor individuals. The reasons for the age-dependent decrease in the responsiveness to IL-1β are not clear, but they could relate to the number of functional IL-1 receptors, or conversely, could reflect induction of receptor antagonists in the cultures.

As indicated above, TNF-α has been shown previously to decrease type I collagen gene expression and to antagonize the TGF-β upregulation of type I collagen gene expression [26–29]. In contrast, TNF-α markedly enhanced type VII collagen gene expression, as detected by the presence of elevated α1(VII) collagen mRNAs. However, this response was noted only in younger donors. Thus, both IL-1β and TNF-α demonstrate a similar pattern of upregulation of type VII collagen gene expression. These data may suggest similar *trans*-activation pathways and/or shared responsive *cis*-elements in the α1(VII) collagen promoter region.

In conclusion, type VII collagen gene expression is clearly subject to modulation by the cytokine network, and the interactions of various growth factors and cytokines may play a role in physiologic conditions, such as anchoring fibril assembly during fetal development, or in repair processes, such as formation of a stable dermal-epidermal junction during epidermal wound healing. Furthermore, these observations suggest pharmacologic potential for cytokines either alone or in combination to correct states characterized by deficient deposition of type VII collagen and the anchoring fibrils in diseases, such as certain forms of dystrophic epidermolysis bullosa.

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