Transforming Growth Factor-β Stimulates Collagen VII Expression by Cutaneous Cells In Vitro

Adrian König and Leena Bruckner-Tuderman

Department of Dermatology, University Hospital Zurich, 8091 Zurich, Switzerland

Abstract. Collagen VII, the major component of cutaneous anchoring fibrils is expressed at a low level by normal human keratinocytes and fibroblasts in vitro. In cocultures of these two cell types, signals from fibroblasts enhance expression of collagen VII by keratinocytes and vice versa. In this study, the effects of a possible mediator of such a stimulation, transforming growth factor-β (TGF-β), were investigated. Its effect on the expression and deposition of the highly insoluble collagen VII was assessed in a semiquantitative manner by a newly developed enzyme-linked immunosorbent assay which is based on immunoblotting. In keratinocyte monocultures, 0.5–20 ng/ml of TGF-β induced a dose-dependent stimulation of collagen VII expression as measured per microgram of DNA. The maximal enhancement was about sevenfold compared to controls. The effect of TGF-β was observed already after 12 h, with a steady increase at least up to 3 d. As previous studies have implicated, untreated cocultures of keratinocytes and fibroblasts exhibited a higher basic level of collagen VII expression, which could be further stimulated about twofold by TGF-β. Fibroblasts alone synthesized very minor quantities of collagen VII and could be only weakly stimulated by TGF-β. This growth factor seems a specific enhancer of collagen VII since the expression of laminin, collagen IV, as well as total protein was increased to a much lesser extent. Our data suggest that TGF-β may be an important mediator of epithelial–mesenchymal interactions and may regulate the synthesis of the anchoring fibrils at the skin basement membrane zone.

The dermo–epidermal junction of the skin represents an epithelial–mesenchymal interface with a unique structure and highly specialized functions (Palade and Farquhar, 1965; Bruns, 1969; Briggaman and Wheeler, 1975a; Tidman and Eady, 1984; Burgeson, 1987). One of its main functions is to provide the resistance of the skin against shearing forces which requires strong cohesion of the skin layers. This is achieved by several interconnected macromolecular networks which attach the epidermis to the basement membrane and to the underlying dermal connective tissue (Timpl, 1989; Burgeson et al., 1990; Yurchenko and Schittny, 1990). One of the major structures mediating the attachment is the anchoring fibril network that extends from the basement membrane to the anchoring plaques in the papillary dermis (Sakai et al., 1986; Keene et al., 1987). Abnormalities of the anchoring fibrils lead to separation of the epidermis from the dermis and to clinical blistering of the skin, as seen in dystrophic epidermolysis bullosa, a group of inherited blistering disorders (Briggaman and Wheeler, 1975b; Hashimoto et al., 1976; Tidman and Eady, 1985; Heagerty et al., 1986; Bruckner-Tuderman et al., 1989, 1991a), or in epidermolysis bullosa acquisita, an inflammatory autoimmune bullous disease (Woodley et al., 1988).

Collagen VII is the major structural component of the anchoring fibrils (Sakai et al., 1986; Burgeson et al., 1990). The structure, biosynthesis and supramolecular assembly of this collagen are known only in part (Lunstrum et al., 1986; Burgeson et al., 1990; Bächinger et al., 1990; Parente et al., 1991), and the regulation of these events remains to be elucidated. Epidermal cells appear the main site of collagen VII production, but their biosynthetic activity is under dermal control (Regauer et al., 1990; König and Bruckner-Tuderman, 1991). In cocultures of keratinocytes and fibroblasts, expression of collagen VII is enhanced as compared to monocultures of either cell type, and in three-dimensional skin equivalent cultures collagen VII can be detected in the epidermal, but not dermal cells (König and Bruckner-Tuderman, 1991).

The nature of the mesenchymal–epithelial signals mediating the stimulation of collagen VII expression in cocultures remains unknown. Direct cell–cell contacts seem less likely because epithelial cells express collagen VII in skin equivalents in which no physical contact between fibroblasts and keratinocytes exists. In contrast, growth factors seem likely candidates as mediators of at least some signals, since their effects on cellular behaviour and production of the extracellular matrix, and their interactions with the matrix have be-
come known in many in vitro systems (for review, see Nathan and Sporn, 1991). A cytokine with a broad spectrum of effects on extracellular matrix is the transforming growth factor-β (TGF-β) peptide family (for reviews, see Roberts et al., 1988, 1990; Massagué, 1990). In addition to controlling cell growth, differentiation, and function, it has been shown to play an important role in remodelling of tissues by affecting the expression of, e.g., many collagens, fibronectin, elastin, matrix metalloproteinases, or their inhibitors (Roberts et al., 1988, 1990; Massagué, 1990).

In this study we show that TGF-β; stimulates in a dose-dependent manner the expression of collagen VII, a major component of the dermo-epidermal junction and a structural protein of the anchoring fibrils. The effect on the expression of collagen VII is significantly larger than on other basement membrane zone proteins such as laminin or collagen IV.

Materials and Methods

TGF-β

Recombinant human TGF-β; was a kind gift from Drs. K. Müller and N. Ceriotti (Ciba-Geigy AG, Basel, Switzerland). It was solubilized in 10 mM HCl and 10% ethanol at a concentration of 0.1 mg/ml. This stock solution was diluted with PBS containing 0.05% BSA to a final TGF-β2 concentration of 200 ng/ml.

Cell Cultures

Human skin fibroblasts were initiated from primary explants and grown in DMEM supplemented with 10% FCS, 4 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Gibco Laboratories, Grand Island, NY). Human keratinocytes were released from skin by trypsination and cultured in serum-free keratinocyte growth medium (KGM) containing 0.09 mM calcium (Boyce and Ham, 1983), 50 µg/ml bovine pituitary extract (BPE) and 5 ng/ml recombinant human EGF (Gibco Laboratories). Cells from the first or second passage were used in the experiments. For cocultures, fibroblasts were seeded at a density of 8,000 cells/cm² in DMEM with 10% FCS. 24 h later, the medium was replaced with KGM, and keratinocytes were added at a density of 40,000–80,000 cells/cm². Cylindroma cells were initiated and cultured in KGM as previously described (Bruckner-Tuderman et al., 1991b). Before assessing collagen VII expression, serum or BPE were omitted from the media for at least 48 h. For the same time period, 50 µg/ml ascorbate was added to the cultures.

Treatment of Cell Cultures with TGF-β2

Keratinocytes, fibroblasts, cocultures, or cylindroma cells were cultivated to semi-confluency in complete culture medium. TGF-β2 treatment was carried out in serum- and BPE-free medium supplemented with 50 µg/ml ascorbate. Appropriate amounts of TGF-β2 stock solution (see above) were added to the medium once, and the medium was not renewed during the incubation period. The incubation period was 48 h for IF experiments and 72 h for the measurements with the enzyme-linked immunoassay. To control cultures, corresponding amounts of PBS containing 0.05% BSA (TGF-β2 solvent) were added for the incubation period.

Antibodies

For detection of collagen VII, polyclonal affinity-purified antibodies to the triple helical domain of human collagen VII were used (Bruckner-Tuderman et al., 1987). The mAb to human collagen IV was kindly provided by Dr. B. Odermatt, Department of Pathology, University of Zurich (Odermatt et al., 1984), and the polyclonal antibodies to human laminin were a generous gift from Dr. M. Paulsson, M. E. Müller Institute for Biomechanics, University of Berne (Paulsson et al., 1987). FITC-labeled antirabbit and antimouse antibodies were purchased from Dakopatts (Glostrup, Denmark). Peroxidase-labeled goat antirabbit antibodies were obtained from Kirkegaard & Perry (Gaithersburg, MD).

Indirect Immunofluorescence Staining (IF)

Cells cultured on cover slips or on plastic Lab-Tek® chamber slides (Nunc Inc., Naperville, IL) were washed twice with TBS, and permeabilized and fixed with 100% methanol for 15 min at −20°C. Incubation with the first antibody was performed overnight and with the FITC-conjugated secondary antibody for 1 h.

Protein and DNA Determination

The total cell culture proteins were precipitated with 10% TCA and dissolved in 1 M NaOH. The protein concentration was measured by a modified Lowry-assay (Lowry et al., 1951; Hudson and Hay, 1989) using BSA for calibration. The DNA content of the cultures was measured according to Labarca and Paigen (1980).

Enzyme-linked Immunoassay for Collagen VII

Due to an extensive insolubility of the tissue form of collagen VII in physiological solutions, common quantitative immunoassays such as ELISA or RIA were not reproducible. A new solid phase immunoassay based on quantitative extraction of collagen VII with denaturing and reducing agents, immunoblotting and measurement of the antibody-bound enzyme activity with an water soluble chromophore substrate was developed.

Cells were cultured in 25-cm² flasks to early confluency and treated with TGF-β2 according to the experimental protocol. After the incubation period, the medium was harvested and proteinase inhibitors including 1 mM phenylmethyl-sulfonlfluoride, 10 mM EDTA, 20 mM N-ethylmaleimide, and 100 mM e-aminoacproic acid were added. Before further processing, the medium was stored on ice. The cell layer was washed twice with ice-cold TBS, and homogenized in cold distilled water by sonication. After aliquots had been separated for DNA assays, the cells were combined with the medium and precipitated with 75% ethanol on ice for 1 h. After centrifugation at 17,000 g for 20 min, the pellet was dissolved in 500 µl of a buffer containing 8 M urea, 2% SDS, 0.1 M 1,4-dithioerythritol and 0.1 M Tris-HCl (pH 6.8). After heating at 100°C for 5 min, the extract was extensively dialyzed against SDS-PAGE sample buffer containing 0.8 M urea, 0.1 M Tris, pH 6.8, 2% SDS, 0.002% bromphenol blue, and 5% glycerine. The volume of all samples was adjusted to the largest volume by adding sample buffer, usually to 750 µl. 50 or 100 µl of each extract was separated by SDS-PAGE (Lämmli, 1970) under reducing conditions, with a 4.5–15% polyacrylamide gradient gel. This was followed by electrotransfer of the proteins onto nitrocellulose (Towbin et al., 1979). To ensure a complete transfer, 0.1% SDS was added to the transfer buffer. The lanes on the filter were marked after staining with 0.2% Ponceau S Red (Sigma Chemical Co., St. Louis, MO) in TBS. After blocking with 2% defatted milk powder in PBS for 30 min, the nitrocellulose sheet was reacted overnight with affinity-purified rabbit antihuman collagen VII antibodies (Bruckner-Tuderman et al., 1987). Washing with PBS was followed by incubation with peroxidase-labeled goat anti-rabbit IgG antibody for 2 h.

After the reaction with the antibodies, lanes with collagen VII standards were cut out and reacted with the water-insoluble substrate 4-chloro-1-napthol. The migration position of collagen VII on the nonreacted nitrocellulose was marked with help of these standards, and the corresponding bands were excised and transferred to test tubes. They were incubated in 0.5 ml of a substrate solution containing 0.03% o-phenylenediamine, 25 mM citric acid, 50 mM NaHPO4, and 0.01% hydrogen peroxide for 15 min. The reaction was stopped by adding 100 µl of 2 M sulfuric acid. The nitrocellulose stripes were transferred into new tubes, and residual bound substrate was eluted with 200 µl of N,N-dimethyl formamide. Both chromophore solutions were combined, clarified by short centrifugation in a microfuge, and the absorbance was read at 492 nm.

For calibration, 5–30 µl of collagen VII containing skin extract were run on the same gel and measured in the same assay, parallel with the test samples. Due to the semiquantitative nature of this solid-phase immunoassay, arbitrary units for type VII collagen were defined. One unit corresponded to the amount of collagen VII present in 1 µl of dermis extract that was prepared by using 400 µl of extraction buffer per cm² of skin surface, as described (Stanley et al., 1985; Bruckner-Tuderman et al., 1987). Application of 5–30 µl of skin extract as a standard resulted in a linear response in the assay. Fig. 1 (4) shows such a standard series stained with a water-insoluble
peroxidase substrate, 4-chloro-l-naphtol, and B shows the corresponding absorbances of the reaction with o-phenylenediamine as a water-soluble substrate. All samples were measured in the linear range of the assay.

**Results**

For initial observation of the effects of TGF-β on collagen VII expression in vitro, small cultures on coverslips were treated with TGF-β2 and subjected to IF. Semiconfluent keratinocytes, fibroblasts, cocultures, or cylindroma cells were cultured for 2 d in serum- or BPE-free medium which was supplemented with 20 ng/ml TGF-β2 and 50 μg/ml ascorbate before processing for IF. In controls, the media were supplemented with ascorbate and PBS containing 0.05% BSA but no TGF-β2.

In dermal fibroblasts, the basal expression of collagen VII was very low (Fig. 2a), and incubation with TGF-β2 induced only a discrete increase of immunoreaction (Fig. 2b). In contrast, the expression of collagen VII by keratinocytes could be significantly stimulated by TGF-β2 (Fig. 2c and d). In cocultures, the basal expression of collagen VII was stronger than in either cell type alone (Fig. 2e), but could still be enhanced by TGF-β2 to some extent (Fig. 2f). As we have shown previously (Bruckner-Tuderman et al., 1991b), epithelial cells derived from a benign skin tumor, the cylindroma, expressed collagen VII at a relatively high level (Fig. 2g). Also in these "high producers," an enhancement by TGF-β2 was observed (Fig. 2h). For preliminary assessment of the degree of stimulation, the response of the cells to different concentrations of TGF-β2 was estimated by visual scoring of the IF staining (Table 1). Addition of 1-30 ng/ml of TGF-β2 resulted in a very weak stimulation in fibroblasts, whereas a clear dose-dependent response was observed in keratinocytes, cocultures, and cylindroma cells. In control experiments, collagen VII was similarly expressed in keratinocytes and cocultures when BPE was included in the medium (data not shown).

The subjective impression of increased collagen VII expression in TGF-β2-treated cultures was confirmed with immunoblotting of culture extracts. Small quantities of collagen VII could be extracted from untreated 25-cm² subconfluent keratinocyte cultures (Fig. 3, lane 2). However, significantly higher amounts were found in cultures incubated with 5 ng/ml TGF-β2 for 3 d (Fig. 3, lane 3). In all extracts, the intact tissue form of collagen VII was present, and no degradation products were observed.

For semiquantitative dose-response measurement, keratinocytes and cocultures were incubated with 0.1-20 ng/ml TGF-β2 for 3 d. The culture extracts were subjected to the solid-phase immunoassay, and the collagen VII content was assessed per microgram of DNA. In control keratinocytes (Fig. 4A), the collagen VII level corresponded to ~1.0 U/μg DNA (one arbitrary unit was defined as the amount of collagen VII present in 1 μl of dermal basement membrane zone extract, as described in Materials and Methods). TGF-β2 induced a dose-dependent stimulation which was about fourfold with 0.5 ng/ml and about sevenfold with 20 ng/ml TGF-β2. A similar dose-response was found in cocultures (Fig. 4B). As expected from the preliminary experiments with IF staining, the basal expression of collagen VII in these cultures was higher, ~5 U/μg DNA. It could be stimulated ~1.5-fold with 0.5 ng/ml, and ~2-fold with 20 ng/ml TGF-β2.

In time-course experiments, a slight effect of TGF-β2 on collagen VII expression was seen after 12 h incubation, and a clear stimulus was measured after 24 h (Fig. 5), with a steady increase up to 72 h.

TGF-β2 was found to stimulate to a lesser extent the expression of two other basement membrane zone proteins, laminin and collagen IV (Table II). As assessed by IF and visual scoring, the basal expression of laminin in keratinocytes was prominent but could still be stimulated by TGF-β2 in a dose-dependent way. When stained with antibodies to collagen IV, keratinocytes showed an immunofluorescence signal that was indistinguishable from the negative controls. Incubation with TGF-β2 induced a barely visible staining for collagen IV.

Total protein content of the keratinocyte cultures treated with up to 5 ng/ml TGF-β2 for 3 d increased by 20-25%, significantly less than the content of collagen VII. As expected from the well-known inhibitory effect of TGF-β on keratinocyte proliferation (Shipley et al., 1986), the DNA content in cultures treated with 0.5-20 ng/ml TGF-β2 for 3 d was 25-30% lower than in control cultures.

**Discussion**

In the present study we demonstrate that TGF-β2 increases in a dose-dependent manner the expression of collagen VII.

| TGF-β2 (ng/ml) | Fibroblasts | Keratinocytes | Cocultures | Cylindroma Cells |
|---------------|-------------|---------------|------------|-----------------|
| 0             | +/-         | +             | ++         | ++              |
| 1             | +/-         | +             | +++        | +++             |
| 5             | +/-         | +             | +++        | +++             |
| 10            | +/-         | +             | +++        | +++             |
| 20            | +           | +++           | ++++       | ++++            |
| 30            | +           | +             | +          | +               |

Indirect immunofluorescence staining of cells grown on cover slips was scored visually. +/-, questionable staining; +, moderate staining; ++, prominent staining; ++++/++++, strong staining.

Figure 1. Enzyme-linked solid phase immunoassay for collagen VII. The tissue form of collagen VII was detected in 5-30 μl of dermal extract by conventional immunoblotting (a) or by the assay using a soluble chromophore substrate (b). For calibration of the assay, the same amounts of dermal extract as in a were used as standards. One arbitrary unit of collagen VII corresponds to the amount present in 1 μl of dermal extract, prepared as described in Materials and Methods.
by cutaneous cells. The quantitation of collagen VII in tissues or in cell cultures has been difficult in the past, due to a tendency to aggregation and subsequent insolubility of the protein, a fact that has impaired studies on regulation of this collagen. To circumvent this problem, a new enzyme-linked immunosassay was utilized here. It is based on immunoblotting and the use of a water-soluble chromophore substrate, and allows semiquantitative assessment of collagen VII which can be solubilized under denaturing and reducing conditions. The assay is linear over a wide range of antigen concentration, and shows small interassay variation. This method can be adapted for any other protein which cannot be assessed with ELISA or RIA due to insolubility or tendency to aggregation.

In vitro, collagen VII can be synthesized by both fibroblasts and epithelial cells (Stanley et al., 1985; Lunstrum et al., 1986; Bruckner-Tuderman et al., 1987; König and Bruckner-Tuderman, 1991). It is unclear, however, which cells produce collagen VII and deposit anchoring fibrils in situ, and how these processes are regulated. Recent studies

Figure 2. Enhanced collagen VII expression in TGF-β-treated cells. Immunofluorescence staining with antibodies to collagen VII of fibroblasts (a and b), keratinocytes (c and d), cocultures (e and f), and cylindroma cells (g and h). The cultures were incubated with 20 ng/ml of TGF-β and ascorbate in serum- or BPE-free medium for 3 d (b, d, f, and h) as described in Materials and Methods. Control cultures (a, c, e, and g) were incubated with ascorbate alone. Bars, 10 μm.
collagen VII expression in cocultures is mutual as the expression can be expected. In other cell systems, the two subtypes of TGF-β exert similar effects mediated by shared receptors which bind TGF-β1 or TGF-β2 for 12-72 h in the presence of ascorbate (●). The growth factor was omitted from parallel control cultures (●). A clear stimulation of collagen VII expression was visible after 24 h, with a steady increase up to 72 h. Collagen VII content of the cultures was assessed by the enzyme-linked immunoassay as described in Materials and Methods. Results are expressed as arbitrary units of collagen VII per microgram of DNA. Each dot represents the value obtained from one culture flask. At 24 h, two dots are superimposed.

Table II. Effect of TGF-β on Expression of Collagen IV and Laminin by Keratinocytes In Vitro

| TGF-β (ng/ml) | Laminin   | Collagen IV |
|--------------|-----------|------------|
| 0            | ++        | −          |
| 1            | +++       | −          |
| 5            | ++++      | +/−        |
| 10           | ++++      | +/−        |
| 20           | ++++      | +/−        |

Indirect immunofluorescence staining of cells grown on cover slips was scored visually. −, no staining; +/−, questionable staining; ++, prominent staining; ++++/+++++, strong staining.
both subtypes (Massagué, 1990), and fibronectin production is stimulated by both subtypes but stronger by TGF-β1 (Hashiro et al., 1991).

The response of normal, low passage skin cells to TGF-β2 in the present study was comparable to observations made in other cell culture systems where 0.5–12.5 ng/ml of TGF-β stimulated ~6–10-fold the expression of fibronectin, laminin (Ignjotz and Massagué, 1986, Vollberg et al., 1991, Hashiro et al., 1991) and other collagens (Varga et al., 1987, Rossi et al., 1988, Madri et al., 1988, Vollberg et al., 1991). This suggests that TGF-β in picomolar concentrations regulates the expression of extracellular matrix components, in particular collagen VII, by keratinocytes and fibroblasts along the cutaneous basement membrane zone. Both human fibroblasts and keratinocytes are capable of synthesizing and secreting TGF-β (Lawrence et al., 1984; Partridge et al., 1989) and they possess specific receptors for this factor (Tucker et al., 1984). It thus seems likely that TGF-β is responsible for at least part of the stimulation of collagen VII observed in cocultures of keratinocytes and fibroblasts.

However, the fact that fibroblasts in monolayer culture can be stimulated with TGF-β2 to express collagen VII to a lesser extent than fibroblasts that are cocultured with keratinocytes, points to the existence of additional keratinocyte-derived mediators that regulate mesenchymal-epithelial interactions in vitro. Potential candidate factors include FGF, PDGF, and EGF which modulate connective tissue metabolism, e.g., during reparatory processes and wound healing (Nanney, 1990; Mustoe et al., 1991). The role of such factors in the regulation of mesenchymal-epithelial interactions in skin and of biogenesis of the anchoring fibrils is being presently investigated in our laboratory.

The precise mechanisms of TGF-β action on expression of collagen VII remain unknown. In other instances, stimulation of collagen gene transcription via nuclear factor 1 binding (Rossi et al., 1988), or preferential stabilization of collagen mRNA (Raghow et al., 1987) have been reported. On the other hand, TGF-β represses matrix metalloproteinase expression and activity (Woessner, 1991), an effect which could lead to accumulation of a protein in the matrix.

In many dermatologic disorders with blistering tendency, abnormal structure and function of the anchoring fibrils can be accompanied by secondary proteolysis. In genetic diseases such as dystrophic epidermolysis bullosa, synthesis of collagen VII is impaired, the dermo-epidermal coherence is destroyed, and the dermal connective tissue is often evident (Hashimoto et al., 1976; Bruckner-Tuderman et al., 1989, 1990). Another example is found in healing burn wounds, where blisters tend to form very easily in spite of complete re-epithelialization of the wound. In such wounds, the globular domains of the anchoring fibrils are present but the collagenous domains are missing, probably due to excessive collagenolytic activity in the dermal granulation tissue (D. Woodley, E. Bauer, and L. Bruckner-Tuderman, manuscript in preparation). In pathologic situations like the above, TGF-β may exhibit therapeutic potential due to its multiple regulatory effects on the extracellular matrix, i.e., stimulation of collagen VII but inhibition of collagenase and stromelysin expression, as well as stimulation of specific matrix metalloproteinase inhibitors (Weber, 1989; Massagué, 1990; Woessner, 1991). Profound understanding of the effects of growth factors and cytokines on the biology of the skin basement membrane zone is essential for planning therapeutic regimens utilizing TGF-β or other biologically active factors.

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