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# Differerential cytokine modulation of the genes LAMA3, LAMB3, and LAMC2, encoding the constitutive polypeptides, α3, β3, and γ2, of human laminin 5 in epidermal keratinocytes

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**Abstract** **Laminin 5, an anchoring filament protein previously known as nicein/kalinin/epiligrin, consists of three polypeptide chains, α3, β3, and γ2, encoded by the genes LAMA3, LAMB3, and LAMC2, respectively. The expression of laminin 5 was detected by Northern hybridization with specific cDNA probes in various epidermal keratinocyte cultures, whereas no expression of any of the three genes could be detected in foreskin fibroblast cultures. Transforming growth factor-β (TGF-β) enhanced LAMA3, LAMB3, and LAMC2 gene expression in human epidermal keratinocytes, as well as in HaCaT and Balb/K cells in culture, although the extent of enhancement was greater for LAMA3 and LAMC2 genes than for LAMB3. Interestingly, tumor necrosis factor-α, (TNF-α) alone did not alter the expression of LAMB3 and LAMC2 genes in human epidermal keratinocytes, whereas it inhibited the expression of LAMA3. These results suggest that the expression of the three genes encoding the laminin 5 subunits is not coordinately regulated by the cytokines tested.**

*Key words:* Keratinocyte; TGF-β; TNF-α; Laminin 5; Gene expression

## 1. Introduction

Laminin 5, a member of the laminin superfamily of proteins comprised of at least seven isoforms, is composed of three polypeptide chains, α3, β3 and γ2, encoded by the LAMA3, LAMB3, and LAMC2 genes, respectively [1]. The expression of laminin 5, previously known as nicein/kalinin/epiligrin [2–4], is largely restricted to the basement membrane zone of the epidermis and other stratified squamous epithelia. Laminin 5 is involved in the attachement of keratinocytes to the basement membrane within the dermal-epidermal junction [3] through interactions with α3β1 and α6β4 integrins [4,5], and it therefore facilitates epithelial-mesenchymal cohesion in vivo [6]. The expression of the laminin 5 genes is diminished in the skin of patients with junctional forms of epidermolysis bullosa (JEB), an autosomal recessive disease characterized by blister formation within the dermal-epidermal basement membrane of the skin [2,7,8], suggesting that the three genes encoding laminin 5 may serve as candidate genes in JEB [8]. Recently, full-length cDNAs for the β3 [9] and the γ2 chains [10], and partial cDNA sequences for the α3 chain [11] have been reported, and recent work has shown that pathogenetic mutations in the genes encoding laminin 5 (LAMA3, LAMB3, and LAMC2) underlie the forms of JEB [12–17].

In attempts to identify cytokines potentially capable of upregulating the expression of laminin 5, we have examined the effects of TGF-β and TNF-α on the expression of the genes encoding the three subunits of laminin 5 in keratinocytes in culture.

## 2. Materials and methods

### 2.1. Cell cultures

Human epidermal keratinocytes, obtained by explanting foreskin specimens, were grown in serum-free, low calcium (0.15 mM), keratinocyte growth medium supplemented with epidermal growth factor, hydrocortisone, insulin and bovine pituitary extract (KGM, Clonetics Corp., San Diego, CA), and utilized in passage 1 to avoid differentiation inherent to subculturing. One hour prior to the experiments, the confluent keratinocyte cultures were placed in fresh KGM.

Human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskin, were utilized in passages 3 to 8. Fibroblasts were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics (50 μg/ml streptomycin, 200 U/ml penicillin-G, 0.25 μg/ml Fungizone). One hour prior to the addition of growth factors, the confluent fibroblast cultures were rinsed with DMEM and placed in DMEM containing 1% FCS.

Mouse Balb/K cells were grown in DMEM supplemented with 10% FCS and 5 ng/ml of murine epidermal growth factor (Clonetics Corp.). One hour prior to the addition of growth factors, the confluent fibroblast cultures were rinsed with DMEM and placed in DMEM containing 1% FCS.

### 2.2. Cytokines/growth factors

Human recombinant TGF-$\beta_2$ was a generous gift from Dr. David R. Olsen, Celtrix Laboratories, Santa Clara, CA. Throughout the text, it will be referred to as TGF-β. Human recombinant TNF-α was purchased from Boehringer Mannheim, Indianapolis, IN.

### 2.3. Northern analyses

Cell cultures were incubated with the cytokines as indicated in the Results section, and total RNA was isolated at the end of the incubations, as previously described [18]. RNA was fractionated in 0.8% agarose gels containing formaldehyde, and analyzed by Northern hybridization with $^{32}$P-labeled cDNA probes [19]. The [$^{32}$P]cDNA–mRNA hybrids were visualized by autoradiography, and the steady-state levels of mRNA were quantitated by scanning densitometry using a He-Ne laser scanner at 633 nm (LKB Produkter, Bromma, Sweden). The following cDNAs were used: for detection of the mRNA encoding the α3 chain, a 1.0 kb human cDNA was synthesized by RT-PCR of total human keratinocyte mRNA, using primers based on the published

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*Abbreviations:* DMEM, Dulbecco's Modification of Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KGM, keratinocyte growth medium; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.


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LAMA3 sequence [11], and subcloned into the TA vector (InVitrogen, San Diego, CA); for the $\beta$3 chain, a 1 kb human cDNA fragment, Kal45, [9], kindly provided by Dr. R.E. Burgeson, Cutaneous Biology Research Center, Charlestown, MA; and for the $\gamma$2 chain, a 0.85 kb human cDNA fragment [10], kindly provided by Dr. Karl Tryggvason, University of Oulu, Oulu, Finland. A rat glyceraldehyde-3-phosphate dehydrogenase cDNA [20] was used as a control.

## 3. Results and discussion

In order to analyze the effects of TGF-$\beta$ on the expression of the genes encoding laminin 5, confluent primary cultures of human epidermal keratinocytes were incubated with various concentrations (0, 0.1, 1, and 10 ng/ml) of TGF-$\beta$, over a 24 h period. Total RNA was extracted and Northern analyses were performed using $^{32}$P-labeled cDNA probes for each of the laminin 5 chain mRNA transcripts. Autoradiograms of the hybridizations, shown in Fig. 1A, indicated that TGF-$\beta$ induces the expression of the three genes encoding the subunit polypeptides of laminin 5, LAMA3, LAMB3, and LAMC2, in a dose-dependent manner. By contrast, hybridization of the same blot with a glyceraldehyde-3-phosphate dehydrogenase cDNA (GAPDH) (Fig. 1, lower panel) indicated that the levels of the corresponding mRNA remained relatively unchanged upon TGF-$\beta$ treatment. Quantitation of the TGF-$\beta$ effect by scanning densitometry (Fig. 1B) revealed that the enhancement of expression of the three laminin 5 genes was not identical. Specifically, LAMA3 gene expression was increased ~5-fold in cells incubated with 0.1 ng/ml of TGF-$\beta$ and up to ~50-fold in the presence of 10 ng/ml of TGF-$\beta$. Similarly, the expression of LAMC2 was increased from ~3- to 40-fold under the same experimental conditions. In contrast, the expression of LAMB3 was almost unaltered by the lowest concentration of TGF-$\beta$ used, and maximal stimulation was only ~6-fold. This variation in the extent of stimulation of the expression of the three laminin 5 subunit genes upon TGF-$\beta$ treatment was consistently observed in keratinocyte cultures established from the skin different donors. These data suggest that the regulation of the expression of the three genes encoding laminin 5 subunits is not tightly co-regulated. A somewhat similar phenomenon has been described for the regulation of the heterotrimeric type VI collagen gene expression by TGF-$\beta$. Specifically, TGF-$\beta$ increases the synthesis of type VI collagen by dermal fibroblasts in culture. This effect is accompanied by an increase in $\alpha$3(VI) collagen mRNA levels whereas the levels of $\alpha$1(VI) and $\alpha$2(VI) mRNAs remain unchanged upon TGF-$\beta$ stimulation [21]. These data suggest that the regulation of $\alpha$3(VI) collagen gene expression is critical for the control of collagen type VI synthesis.

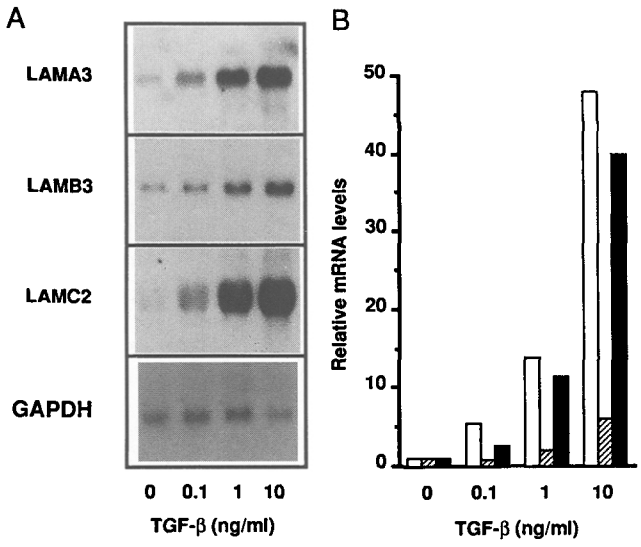


Fig. 1. Effect of TGF-$\beta$ on laminin 5 gene expression in cultured human epidermal keratinocytes. Confluent primary cultures of epidermal keratinocytes were incubated in serum-free KGM without or with TGF-$\beta$ (0.1, 1 or 10 ng/ml). After 24 h, total RNA was isolated and analyzed by Northern hybridizations with cDNA probes specific for human LAMA3, LAMB3 and LAMC2 mRNAs. A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as a control (Panel A). The relative levels of expression of the three laminin 5 genes as determined by scanning densitometry of the respective autoradiograms, after correction for the levels of the GAPDH mRNA in the same RNA preparation, is indicated in panel B.

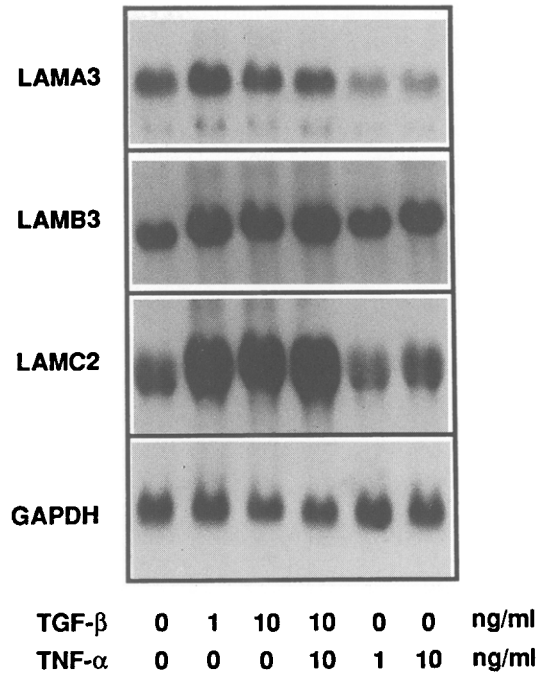


Fig. 2. Effect of TGF-$\beta$ and TNF-$\alpha$, alone or in combination, on laminin 5 gene expression in cultured epidermal keratinocytes. Confluent keratinocyte cultures in serum-free KGM were incubated for 24 h in the absence or presence of TGF-$\beta$ and/or TNF-$\alpha$, in concentrations indicated. After 24 h, total RNA was extracted and analyzed by Northern hybridizations with human LAMA3, LAMB3 and LAMC2 specific cDNAs; a GAPDH cDNA was used as a control.

Similar experiments were carried out utilizing mouse Balb/K and HACAT cells. TGF-$\beta$ enhanced the expression of LAMA3 and LAMC2 to a greater extent than LAMB3 in HaCaT cells (not shown), confirming the observations made using normal epidermal keratinocytes (see above). A weak hybridization signal corresponding to an mRNA of the same size as the human form could be detected with each cDNA probe in RNA preparations from mouse Balb/K cells (not shown). We observed increased expression for all three genes encoding the laminin 5 subunits upon stimulation with TGF-$\beta$. However, quantitation of the growth factor effect was rendered difficult by the low hybridization signal, which may reflect the use of heterologous cDNA probes.

To investigate whether the laminin 5 genes were expressed in dermal fibroblasts, confluent cultures of these cells were incubated in DMEM containing 1% fetal calf serum, in the presence or absence of TGF-$\beta$. RNA extraction followed by Northern analyses did not allow the detection of any of the three laminin 5 genes, even upon TGF-$\beta$ stimulation (not shown), suggesting that their expression in the skin is restricted to basal keratinocytes in the epidermis. The restricted expression of laminin 5 to basal keratinocytes contrasts that of type VII collagen, another component of the cutaneous basement membrane zone, which is expressed at a significant level also in dermal fibroblasts, especially upon growth factor stimulation [22].

We have previously demonstrated that TNF-$\alpha$, a pleiotropic cytokine capable of modulating the expression of a variety of extracellular matrix-related genes (reviewed in [23]), synergizes with TGF-$\beta$ to up-regulate the expression of type VII collagen, whereas it antagonizes the up-regulation of type I collagen gene expression by TGF-$\beta$ [22]. In order to examine whether the expression of laminin 5, and its up-regulation by TGF-$\beta$, could be altered by TNF-$\alpha$, cultures of primary epidermal keratinocytes were incubated for 24 hours in the presence of TGF-$\beta$ or TNF-$\alpha$, either alone or in combination with each other. As shown in Fig. 2, treatment of human keratinocytes by TGF-$\beta$ (lanes 2 and 3, 1 and 10 ng/ml respectively) strongly elevated the expression of LAMA3 and LAMC2, and to a lesser extent that of LAMB3, confirming the data presented in Fig. 1. In contrast, TNF-$\alpha$ alone inhibited the expression of LAMA3, but not that of LAMB3 and LAMC2 (lanes 5 and 6). When TNF-$\alpha$ was added concomitantly with TGF-$\beta$ (lane 4), it had no effect on the response of either gene studied to TGF-$\beta$.

As indicated above, junctional epidermolysis bullosa (JEB) is a group of blistering skin diseases and the characteristic skin fragility is due to reduced laminin 5 expression at the cutaneous basement membrane zone [7]. Low levels of laminin 5 expression attest to the critical role of this anchoring filament protein in providing integrity to the dermal-epidermal junction. Thus, up-regulation of the genes encoding laminin 5 polypeptides could be expected to ameliorate the clinical manifestations in these patients. It should be noted, however, that the limited number of mutations in the three genes encoding laminin 5 disclosed thus far are either nonsense mutations or amino acid substitutions [7], mutations which compromise the assembly of trimeric laminin 5 molecules into functional anchoring filaments. However, in case of putative regulatory mutations in JEB or in physiological situations requiring rapid re-epithelialization, such as in wound healing, one would anticipate that cytokine/growth factor-mediated up-regulation of laminin 5 gene expression could be beneficial.

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