Human Epidermal Keratinocytes Are a Source of Tenascin-C during Wound Healing

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Tenascin-C is a large hexameric extracellular matrix glycoprotein that is expressed in a temporally and spatially restricted pattern associated with stromal-epithelial interactions. In adult human skin, the expression level of tenascin-C is low, but tenascin-C is abundantly present in the dermal compartment during embryogenesis and wound healing and in skin tumors. Herein we have investigated the cellular source of tenascin-C production in human skin, both in vitro and in vivo, by using immunohistochemistry, mRNA in situ hybridization, western blotting, and an enzyme-linked immunoassay. In addition we studied the cell-matrix interaction between epidermal keratinocytes and purified tenascin-C. By using in vitro culture models, we found that keratinocytes not only synthesize and secrete tenascin-C but can also deposit tenascin-C in de-epidermized dermis in a pattern that is very similar to that in vivo. In vivo, during wound healing of normal human skin, we found tenascin-C extracellularly in the wound bed and also in a granular pattern within the neo-epidermis. By mRNA in situ hybridization, we could identify the basal migrated keratinocytes as the main source of tenascin-C in the early phase of wound healing. In the granulation phase, tenascin-C expression by the keratinocytes is downregulated. Cultured keratinocytes were found to adhere poorly to tenascin-C, and those that did adhere retained a rounded morphology. We conclude that human keratinocytes are a major source of tenascin-C during the early phase of wound healing, and we hypothesize that tenascin-C is unlikely to be an adhesive substrate for migrating keratinocytes. Key words: in situ hybridization/epithelial-cell adhesion. J Invest Dermatol 108:776-783, 1997

Tenascin-C is a large hexameric extracellular matrix glycoprotein consisting of subunits of 220-320 kDa that are linked by disulfide bonds (for review see Schenck and Chiquet Ehrismann, 1994; Chiquet Ehrismann et al, 1994; and Lightner, 1994). The tenascin-C subunits have a modular structure, in humans forming a cysteine-rich domain at the N terminus, followed by 14.5 domains with homology to epidermal growth factor, a number of fibronectin type III repeats (varying from 9 to 16 as a result of alternative splicing of tenascin-C RNA), and a fibrinogen-like domain at the C terminus. Recently, two proteins with the same general structure have been described, named tenascin-R (or restrictin) and tenascin-X. In analogy, tenascin is called tenascin-C in more recent literature, where the C stands for cytotactin, a synonym for tenascin. Tenascin-C is abundant during embryogenesis, particularly at mesenchymal-epithelial interaction sites (Cossin et al, 1986; Aufderheide et al, 1987; Aufderheide and Ekbloom, 1988; Ekbloom and Aufderheide, 1989; Vainio et al, 1989; Chuong et al, 1991), but expression is limited in adult tissues. In normal skin tenascin-C is detected in the dermis, where it is sparsely distributed at the dermal—epidermal junction and surrounds blood vessels and epidermal adnexa (Lightner et al, 1989; Schalkwijk et al, 1991b; Shikata et al, 1994). In conditions of epidermal hyper-proliferation, such as psoriasis (Schalkwijk et al, 1991b), epidermal tumors (Stamp, 1989; Schalkwijk et al, 1991b; Verstraeten et al, 1992; Shikata et al, 1994), and injury (Schalkwijk et al, 1991a; Betz et al, 1993; Juhasz et al, 1993; Latijnhouwers et al, 1996), tenascin-C expression in the papillary dermis is strongly upregulated. The role of tenascin-C in vivo is still a matter of debate. In vivo studies have suggested various functions for tenascin-C, including modulation of cell proliferation and cell adhesion (Chiquet Ehrismann et al, 1988; Friedlander et al, 1988; Lotz et al, 1989; Spring et al, 1989; Lightmer and Erickson, 1990; Murphy Ullrich et al, 1991; Prieto et al, 1992, 1993) and suppression of the immune response (Ruegg et al, 1989; Hemesath et al, 1994). Knocking out the tenascin-C gene in mice, however, does not induce marked phenotypic changes. Whether the tenascin-C-like molecules tenascin-R and tenascin-X in these knock-out mice take over the functions that are normally exhibited by tenascin-C is not yet clear. For many tissues, reports point to mesenchymal cells as the cellular source of tenascin-C, whereas epithelial cells induce or modulate tenascin-C production. When mesenchyme is co-cultured with epithelial cells, for example, mesenchymal tenascin-C expression is induced (Aufderheide and Ekbloom, 1988; Inaguma et al, 1988). In vivo, increase in mesenchymal tenascin-C also correlates with epithelial changes, notably epidermal hyper-proliferation in skin (Stamp, 1989; Schalkwijk et al, 1991a, 1991b; Verstraeten et...
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al, 1992; Betz et al, 1993; Juhasz et al, 1993; Shikata et al, 1994; Lartijnhouwers et al, 1996). Consequently, epidermal keratinocytes were considered to merely stimulate tenasin-C production of dermal fibroblasts in skin. More recently, however, epithelial cells were reported to produce tenasin-C as well. Cells may secrete tenasin-C only when cultured in vitro (Kawakatsu et al, 1992) and not after injection in nude mice (Sakai et al, 1993), although the opposite is possible as well (Hiriiwa et al, 1993). Immunohistochemically, tenasin-C was detected in developing corneal epithelium (Kaplon et al, 1991), epithelial lung tumor cells (Sotiri et al, 1993), human amnion cells (Limala et al, 1993), and human breast tissue (Lightner et al, 1994). It should be noted, however, that the extracellular localization of tenasin-C hampers the immunohistochemical identification of cells expressing tenasin-C. In situ hybridization is the preferred approach to identify the cellular source of tenasin-C. By in situ hybridization, tenasin-C mRNA was detected in the epithelium of developing bronchi (Prieto et al, 1990; Koch et al, 1991) in normal and malignant breast epithelium (Lightner et al, 1994) and cornea (Tucker, 1991). In the epithelium of developing chicken feather buds, however, no tenasin-C mRNA was found (Prieto et al, 1990). These findings of epithelial tenasin-C expression led us to further examine tenasin-C expression in skin. Our data demonstrate that epidermal keratinocytes secrete tenasin-C and deposit tenasin-C in the extracellular matrix in vitro and in vivo during wound healing. In addition we investigated the effect of tenasin-C in cell adhesion assays. The results indicate that tenasin-C is a poor adhesive substrate for cultured keratinocytes.

MATERIALS AND METHODS

Cells and Culture Conditions Tenasin-C production was examined in human gliaoblastoma and osteosarcoma cell lines and in primary cultures of epidermal keratinocytes and dermal fibroblasts. The human gliaoblastoma cell lines U87-MG, U138-MG, and U373-MG were provided by R. Verstraeten (Free University of Amsterdam, The Netherlands), and Saos-2 sarcoma cells were a gift from E. Mackie (Royal Veterinary College, London, United Kingdom). The cell lines are available from the American Type Culture Collection (Rockville, MD) as HTB14, HTB16, HTB17, and HTB85, respectively. All cultured cells were tested negative for mycoplasma.

Cell lines and dermal fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM)/F-12, 3:1 (vol/vol), supplemented with penicillin (100 units per ml) plus streptomycin (100 mg per ml) and 10% fetal bovine serum. Human keratinocytes from skin biopsies of healthy donors were initially cultured by the method of Rheinwald and Green (1975). Keratinocytes from these cultures were again seeded on a feeder layer of irradiated 3T3 cells or, alternatively, in serum-free medium. For the method of Rheinwald and Green, DMEM/F-12, 3:1 (vol/vol), supplemented with 0.4 mg hydrocortisone per ml, 1 mg insulin, 10 ng epidermal growth factor per ml, 6% fetal bovine serum, 100 units penicillin per ml, and 100 mg streptomycin per ml was used. Serum-free culture medium was keratinocyte growth medium (KGM). KGM was composed of keratinocyte basal medium (Clonetics, San Diego, CA; 0.15 mM calcium) supplemented with 0.1 mM ethanamolamine, 0.1 M phosphothionolamine, 10 ng epidermal growth factor per ml, 5 mg insulin per ml, 0.4% (vol/vol) bovine pituitary extract (Clonetics, San Diego, CA), 0.5 mg hydrocortisone per ml, 100 units penicillin per ml, and 100 mg streptomycin per ml.

Before reaching confluence, the serum concentration of serum-containing medium was lowered to 1%. After 3 d, medium was collected and tenasin-C concentrations were determined by an enzyme-linked immunosorbent assay (ELISA).

In Vitro Reconstruction of Epidermis on De-Epidermized Dermis (DED) Keratinocytes cultured by the method of Rheinwald and Green were seeded on DMEM/F-12, 3:1 (vol/vol), supplemented with 5% HyClone newborn bovine serum (Greiner, Alphen a/d Rijn, The Netherlands), 0.4 mg hydrocortisone per ml, 1 mM insulin, 5 mg streptomycin per ml, and 5 mg insulin per ml. Cultures were maintained submerged in the medium for 3 d and then were air-exposed for another 3–14 d. The medium used for air-exposed culture was supplemented with 10 ng epidermal growth factor per ml. Generation of DED and culture methods have been described previously (Frunerias et al, 1983; Ponec, 1991).

Biopsies Experimental wounds were made by taking partial thickness punch biopsies with a diameter of 3 mm from the upper arm of healthy volunteers. Full-thickness punch biopsies with a diameter of 4 mm were taken from the different wounds after 2, 4, 7, 14, 60, and 365 d. These secondary biopsies, which included the healing wounds and some of the adjacent dermal skin, were embedded in Tissue Tek OCT Compound, frozen, and stored at -80°C until use or were formalin-fixed and embedded in paraffin. Permission for experiments on humans was obtained from the Medical Ethics Committee of the University Hospital in Nijmegen.

Antiserum Antibodies directed against human tenasin-C were the mouse monoclonal antibodies T2H5 (for reference, see Schalkwijk et al, 1991a) and TN2 (Dako, Glosstrop, Denmark) and two rabbit polyclonal antiserum A107 and TN953. A107 was obtained from Chemicon International (Temecula, CA). We raised TN953 in New Zealand White rabbits by immunization with highly purified human tenasin-C from U138-MG cells. Traces of anti-fibronectin reactivity in TN953 were removed by solid-phase absorption with human fibronectin. Antibodies against human fibronectin were a mouse monoclonal antibody (Sigma–Aldrich, Bornem, Belgium) and a rabbit polyclonal antiserum (Life Technologies, Breda, The Netherlands).

Immunohistochemistry T2H5, TN2, and TN953 were used for staining of tenasin-C in cryostat sections. In addition, TN2, TN953, and A107 were applied to detect tenasin-C in formalin-fixed sections. Staining with T2H5 was performed by an indirect immunoperoxidase staining protocol described previously in human melanoma cell lines and melanoma cell lines (Schalkwijk et al, 1991a). For TN2, TN953, and A107, the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used according to the manufacturer’s instructions, with dianinobenzidin as the chromogenic substrate. After immunostaining, sections were counterstained with hematoxylin.

ELISA To quantify tenasin-C concentrations in culture medium, a sandwich-type ELISA was developed that is similar to the one described by Schenk and colleagues (1992). Polystyrene microtiter plates were coated with T2H5. After blocking free protein binding sites with bovine serum albumin (BSA), dilution series of standards, standard, and blanks were incubated in the wells, and A107 was subsequently used to detect bound tenasin-C. A107 binding was assessed with peroxidase-conjugated swine antirabbit and antimouse antibodies to detect bound tenasin-C. The color reaction was stopped by adding H2SO4 and evaluated by reading the absorbance of the color product at 490 nm, with 655 nm as a reference wavelength. The same method was applied for the fibronectin-specific ELISA. For these a mouse monoclonal antibody and a rabbit polyclonal antiserum against human fibronectin were used. Chicken egg ovalbumin was used instead of BSA in the fibronectin ELISA.

The standards used in ELISAs were human tenasin-C purified from the media of 3T3 cultures. The standards used in the ELISA were a mouse monoclonal antibody (Sigma–Aldrich, Bornem, Belgium) and fibronectin from human plasma (0.1% solution, Sigma–Aldrich, Bornem, Belgium). The detection limits were approximately 20 ng tenasin-C per ml or 80 ng fibronectin per ml, respectively.

Construction of Plasmids and Synthesis of RNA Probes Total RNA was isolated from cultured human dermal fibroblasts, and cDNA was synthesized by reverse transcription with oligo(dT) primers. Part of the cDNA was amplified by using a polymerase chain reaction and subcloned in pGEM-3. The tenasin-C-coding insert corresponds to nucleotides 6016–6475 of the human tenasin-C cDNA sequence as submitted by Gherzi and colleagues to the EMBL Database Library (accession number X78565), spanning part of the most C-terminally located fibronectin type III repeat and part of the fibronectin-like domain.

The plasmid was linearized with BaulHI to synthesize an antisense complementary RNA probe by using T7 polymerase and with EcoRI to synthesize control sense complementary RNA by using Sp6 polymerase in the reverse orientation. Dioxigenin (DIG)-labeled anti-sense and sense probes were synthesized by using a Sp6/T7 in vitro RNA synthesis kit in the presence of DIG-UTP (Boehringer Mannheim, Mannheim, Germany). Synthesis was performed at 37°C with T7 polymerase and at 40°C with Sp6 polymerase. The anti-sense probe thus produced was 471 nucleotides long, and the sense probe was 498 nucleotides long.

In Situ Hybridization In situ hybridization was performed on frozen sections as described (Pfundt et al, 1996) with the modification that the 0.1X sodium citrate/chloride buffer used to wash slides after hybridization contained 60% formamide instead of 50%. Optimal probe concentrations were determined by a pilot hybridization in a nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.
Tenascin-C Purification

U138-MG was used for tenascin-C purification. Before the cells reached confluence, the serum concentration of the medium was lowered to 1% fetal bovine serum and cultures were maintained for an additional 2 mo, during which medium was collected every 3 or 4 d for tenascin-C purification. Proteinase inhibitors (2 mM ethylenediamine tetraacetic acid, 0.3 mM phenylmethylsulfonyl fluoride, and 2 μg aprotinin per ml) and 0.05% NaN3 were added to the collected medium.

The medium was subjected to two rounds of anion-exchange chromatography. First, tenascin-C was eluted from hydroxyapatite using buffer A containing 50 mM NaH2PO4, 0.12 M NaCl, 0.01% NaN3, and 0.001% NaN3. The fractions that contained most of the tenascin-C were used for final purification on the Smart System using a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) and a gradient of 0-1 M NaCl in 20 mM Tris(hydroxymethyl)aminomethane (pH 7.9). In this way, tenascin-C could be quantitatively separated from fibronectin (see Fig. 6). The concentration of purified tenascin-C was quantified by ELISA. Qualitative analysis included fibronectin-specific ELISA and western blotting. In addition, the hemagglutinating activity of the tenascin-C preparations was verified (Erickson and Inglesias, 1984; Clüetz Ehrismann et al., 1986). On western blots of reduced tenascin-C, specific antisera stained two tenascin-C bands with molecular weights exceeding 200 kDa. The only contaminating proteins were traces of fibronectin and BSA; laminin was not detected. According to ELISA results, the purified tenascin-C typically contained less than 3% fibronectin.

Cell Adhesion Assays

Adhesive proteins used in the adhesion assays were fibronectin from human plasma (0.1% solution, Sigma-Aldrich, Bornem, Belgium), laminin purified from human placenta (Life Technologies, Breda, The Netherlands), tenascin-C purified from the glioblastoma cell line U251-MG (Chemicon International, Temecula, CA), and human tenascin-C that we purified ourselves from the glioblastoma cell line U138-MG. Polystyrene microtiter plates were coated by incubation overnight at 4°C with 50 μl of a solution containing the appropriate protein diluted in Dulbecco’s PBS (DPBS = PBS containing 0.9 mM CaCl2 • 2H2O and 0.49 mM MgCl2 • 6H2O). Unbound protein was removed by washing with DPBS and free protein binding sites were blocked by incubation for 1 h at 37°C with DPBS containing 1% BSA.

For adhesion assays only secondary cultures of keratinocytes were used. Cells from subconfluent keratinocyte cultures were harvested by trypsinization and subsequently resuspended in DPBS with 5% fetal calf serum to inactivate trypsin. In cases where keratinocytes had been cultured according to the method of Rheinwald and Green, the feeder cells were removed with ethylenediamine tetraacetic acid prior to trypsinization. After two washes in succession with DPBS and DMEM supplemented with 0.25% BSA and 20 mM HEPES (pH 7.4), cells were resuspended in DMEM, 0.25% BSA, and 20 mM HEPES (pH 7.4) and seeded into the wells (5000–10,000 cells per well). Cells were allowed to attach for 2 h at 37°C in a 5% CO2-humidified atmosphere. Non-adherent cells were removed by washing with 200 μl DPBS per well until the cells in the BSA-coated control wells were sufficiently removed as monitored by visual inspection. Typically this took 3 washes. The attached cells were fixed with 3.8% phosphate-buffered formalin and stained with 0.5% toluidine blue. Attached cells were counted directly or quantified by measurement of the absorbance at 655 nm after solubilizing the cell bound toluidine blue in 100 μl cetrime buffer (0.4% cetyltrimethylammonium bromide, 0.1 M Tris(hydroxymethyl)aminomethane (pH 8.5), and 1 mM NaCl). The number of cells was read from a calibration curve that was obtained by plotting the number of cells as determined by direct counting against the absorbance measured.

Phorbol esters can stimulate integrin ligand-binding capacity of integrins, presumably by inducing conformational changes (Diamond and Springer, 1994). In adhesion-stimulation assays, keratinocytes were, therefore, seeded in the presence of 25 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Bornem, Belgium). In inhibition assays, antiserum was used to block the coated adhesive protein before seeding the cells by incubating coated wells with a saturating concentration of the appropriate antiserum in DPBS with 1% BSA for 1.5 h at 37°C.

All assays were performed at least in duplicate, and in individual assays, triplicate or quadruplicate wells were used for each assay condition. Adhesion results were donor-independent as comparable results were obtained with keratinocytes of different individuals. 

RESULTS

Tenascin-C Production by Cultured Cells

To investigate the ability of several cell lines and primary cultures of human skin cells to produce tenascin-C, the tenascin-C concentration in the conditioned medium was assessed by a sandwich ELISA. Results are summarized in Table I. In the unconditioned culture medium, no tenascin-C was detected. Glioblastoma and sarcoma cell lines secreted large amounts of tenascin-C, as was reported previously (Erickson and Bourdon, 1989; Mackie and Tucker, 1992), and of these, U138-MG was chosen as producer cell line for tenascin-C that was used in adhesion assays. Medium of dermal fibroblast cultures contained tenascin-C as well. Remarkably, tenascin-C was also detected in the medium of epidermal keratinocytes, cultured on a feeder layer of irradiated mouse fibroblasts by the method of Rheinwald and Green or in the serum-free medium KGM. Western blot analysis of tenascin-C in the supernatants of cultured keratinocytes under reducing conditions revealed the presence of two immunoreactive bands with molecular weights greater than 200 kDa (data not shown). It should be noted that because of differences in cell number and medium volume in the various cultures, the absolute amounts of tenascin-C produced cannot be compared directly.

Tenascin-C Deposition by Keratinocytes Cultured on DED

We used an in vitro model for reconstructed epidermis to investigate whether tenascin-C is also produced in a culture model that allows full stratification of the keratinocytes and to analyze whether tenascin-C is deposited in the extracellular matrix or is only secreted in the medium. In this model keratinocytes are seeded on DED. The DED is prepared for culture in such a way that no viable cells remain in the tissue. This was confirmed by hematoxylin staining of DED, which demonstrated that no nuclei were present in the DED. In immunohistochemical staining of DED with tenascin-C antiserum (T2H5, TN2, A307, and TN953) was virtually negative. When keratinocytes were seeded on a layer of DED, however, marked tenascin-C staining was observed in the DED, indicating that the keratinocytes produce tenascin-C that is deposited in the DED underneath. Tenascin-C was detected after 3 d of submerged culture, when the cultures resemble a squamous non-keratinizing epithelium. Tenascin-C remained present at the dermal-epidermal junction during subsequent air exposure, which induces terminal differentiation of the keratinocytes as indicated by the formation of a stratum corneum (Fig 1).

Keratinocytes Express Tenascin-C during Wound Healing

In a previous study, we described tenascin-C expression in healing wounds during a 14-d time course and its relationship with epidermal hyper-proliferation (Latijnhouwers et al, 1996). We now have extended the time course to 1 y, and instead of one polyclonal tenascin-C antiserum (i.e., A107), we have now used a panel of monoclonal antibodies and polyclonal antiserum in combination with a more sensitive staining protocol, which indicates that the expression levels of tenascin-C in healing skin wounds are much higher than previously anticipated. In addition to the known increase of tenascin-C expression in the papillary dermis at the wound margins, we noticed a thin discontinuous line of staining beneath migrated keratinocytes. Both with the polyclonal antiserum and the monoclonal antibodies, deposition of tenascin-C in the wound bed was observed beneath the entire sheet of migrating

Table I. Tenascin-C Is Detected in the Conditioned Medium of Tumor Cell Lines and Cultured Normal Skin Cells by ELISA

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>TN-C Concentration (mg/ml)</th>
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<tbody>
<tr>
<td>U87-MG</td>
<td>2.07 ± 0.74</td>
</tr>
<tr>
<td>U138-MG</td>
<td>5.22 ± 0.07</td>
</tr>
<tr>
<td>U373-MG</td>
<td>2.28 ± 0.19</td>
</tr>
<tr>
<td>Sato-2</td>
<td>1.12 ± 0.24</td>
</tr>
<tr>
<td>Dermal fibroblast</td>
<td>1.04 ± 0.13</td>
</tr>
<tr>
<td>Epidermal keratinocyte&lt;sub&gt;a&lt;/sub&gt;</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>Epidermal keratinocyte&lt;sub&gt;b&lt;/sub&gt;</td>
<td>0.21 ± 0.07</td>
</tr>
</tbody>
</table>

* Data are duplicate tenascin measurements (ELISA). Comparable results were obtained for different cultures of the same cell types. TN-C, tenascin-C.

* Cultured according to the method of Rheinwald and Green.

* Serum-free culture in KGM.
DISCUSSION

Upon further analysis, the question of how the interaction between the two components of the reaction, namely the electrontransfer reaction and the conformational changes in the protein, can be better understood. The experimental results obtained suggest that the conformational changes in the protein are induced by the electrontransfer reaction, rather than vice versa.

Further experiments are needed to confirm these findings and to explore the mechanism of the interaction between the two components. It is hoped that this study will provide new insights into the understanding of the electrontransfer reaction and the conformational changes in the protein.
Figure 2. Tenascin-C in serial sections of a human skin wound after 2 d. Tenascin-C expression was assessed by immunohistochemical staining with T2H5 (A) and in situ hybridization with DIG-labeled anti-sense (B) and sense (C) probes. Tenascin-C protein shows a continuous pattern at the dermal-epidermal junction and in addition it is stained beneath migrated keratinocytes. Tenascin-C mRNA is demonstrated in some dermal cells but is most prominent in migrated keratinocytes (B). Sections are counterstained with hematoxylin (A) or methyl green (B,C). Scale bar, 100 μm.

Figure 3. Tenascin-C in serial sections of a human skin wound after 4 d. Tenascin-C protein is detected immunohistochemically with T2H5 at the dermal-epidermal junction of the wound margin, beneath the neo-epidermis, and sporadically in the migrated keratinocytes themselves (A). Nonradioactive in situ hybridization reveals that tenascin-C mRNA is mainly expressed by basally located keratinocytes of the neo-epidermis; dermal cells are only sporadically stained (B). For comparison hybridization obtained with the control sense tenascin-C probe is shown (C). Sections are counterstained with hematoxylin (A) or methyl green (B,C). Scale bar, 100 μm.

Figure 4. Tenascin-C in serial sections of a human skin wound after 7 d. Tenascin-C protein continuously lines the neo-epidermis of the closed wound (A) and is detected within the neo-epidermis. To detect tenascin-C protein immunohistochemical staining with T2H5 was performed, and the section was counterstained with hematoxylin. High levels of tenascin-C mRNA are found in the basally located keratinocytes of the neo-epidermis (B) by in situ hybridization with a DIG-labeled anti-sense probe. As a control, hybridization with the sense tenascin-C probe was performed (C). Sections shown in B and C were counterstained with methyl green. Scale bar, 50 μm.

Figure 5. Tenascin-C in serial sections of a human skin wound after 14 d. Tenascin-C is abundant at the protein level; massive immunohistochemical staining is observed in the granulation tissue (A), and in the neo-epidermis, a marked granular staining is observed (B). In situ hybridization with a DIG-labeled anti-sense probe shows that tenascin-C mRNA (•), however, is only sporadically detected (C). T2H5 was used for immunohistochemical staining (A,B) and sections were counterstained with hematoxylin (A,B) or methyl green (C). Scale bars: (A) 200 μm; (B) 50 μm; (C) 100 μm.
Figure 6. Tenascin-C purification. Silver staining of a 4–20% gradient gel electrophoresed under reducing conditions. Lane 1, culture medium of U138-MG cells; lane 2, fraction eluted from hydroxylapatite; lane 3, fraction of the Mono Q column containing fibronectin and BSA; lane 4, final tenascin-C preparation after separation on the Mono Q column; lane 5, commercially available tenascin-C lane 6, molecular weight markers.

when they are cultured without feeder in serum-free medium, indicating that serum- or feeder-derived factors are not necessary to induce tenascin-C production. Immunohistochemical analysis of reconstructed epidermis showed that keratinocytes in vitro are able to deposit tenascin-C in the extracellular matrix as well. Tenascin-C was detected in cultures that were maintained submerged in the culture medium for 3 d and remained present during subsequent air-exposed culture. Whether this is caused by continued tenascin-C expression or slow turnover of previously made protein cannot be concluded from these immunohistochemical data. Interestingly, the site of tenascin-C deposition, namely the dermo-epidermal junction of the cultures, corresponds to the location of tenascin-C in vivo. These in vitro models, the monolayer keratinocyte culture systems and the reconstructed epidermis, should be useful when further investigating the regulation and effects of epidermal tenascin-C expression.

The finding that keratinocytes express tenascin-C in vitro is clearly relevant for the in vivo situation, as we could detect tenascin-C in the epidermis of healing skin wounds at the protein and the mRNA levels. Epidermal tenascin-C expression was previously demonstrated in feeder-cultured human keratinocytes and in amphibian epidermis (Onda et al., 1991). Recently, Aukhil and colleagues (1996) reported on tenascin-C expression by keratinocytes during healing of incisional wounds in rats where they observed expression patterns similar to what we found in human tissue. In a previous report we concluded that keratinocytes could potentially interact with tenascin-C in the later stages of wound healing but were unlikely to come into contact with tenascin-C in the early phase because of the undetectable levels of tenascin-C under the migrating keratinocytes (Latijnhouwers et al., 1996). We, however, have now used a panel of polyclonal and monoclonal antibodies combined with a much more sensitive staining protocol that indicates that the expression levels of tenascin-C are much higher than previously anticipated. In this study we could detect tenascin-C in the dermis underneath the migrating keratinocytes. In addition, keratinocytes themselves are stained for tenascin-C protein. In situ hybridization results indicate that in the early phase of wound healing, before wound closure, keratinocytes of the neo-epidermis are the major source of tenascin-C. By the time that the wounds are closed, tenascin-C production in the neo-epidermis is accompanied by abundant dermal tenascin-C expression in the granulation tissue. Subsequently mRNA synthesis decreases: at day 14 after wounding very few cells in either the dermis or the epidermis express tenascin-C mRNA. In contrast, tenascin-C protein is most abundant at day 14, indicating that maximum protein

Figure 7. Adhesion to fibronectin and tenascin-C of keratinocytes cultured in KGM or by the method of Rheinwald and Green. Assays were performed with (■) or without (□) PMA. Adhesion without PMA was performed in triplicate or quadruplicate in six experiments, and the effect of PMA was tested six and three times for feeder-cultured and KGM-cultured cells, respectively. Representative data from one of these experiments are shown. Error bars, SEM (n = 3).

Figure 8. Adhesion of feeder-cultured keratinocytes. A comparison is made between adhesion to laminin (LN), fibronectin (FN), commercially available human tenascin-C (TN com), and tenascin-C that we purified from human glioblastoma cells (TN). Proteins were coated at a concentration of 20 µg per ml, except for purified tenascin-C, which was used at 4, 20, and 100 µg per ml. Adhesion without PMA (□) was compared to adhesion in the presence of PMA (■). One representative experiment of six separate experiments is shown. Error bars, SEM [n = 4 (LN and FN) or 3 (TN com and TN)].

levels are reached later than maximum mRNA levels and that the turnover of tenascin-C in the wounds is rather slow.

Because tenascin-C is detected beneath the migrating keratinocytes that do not encounter an intact basal membrane structure (Latanjhouwers et al, 1996), we wondered whether it may function as a kind of provisional matrix to which these keratinocytes adhere. The results of the adhesion assays indicate that keratinocytes adhere poorly to tenasin-C. Therefore, tenasin-C does not seem to be an important adhesive substrate for keratinocytes. A prerequisite for tenasin-C having a direct effect on keratinocytes is interaction between keratinocytes and tenasin-C, which is most likely receptor-mediated. In the adhesion assays, very few keratinocytes remained attached to tenasin-C, suggesting that interactions between such putative receptors on keratinocytes and tenasin-C are not very strong. It should be noted, however, that strong intracellular effects may be evoked in spite of weak receptor binding. This was recently reported for the interaction between fibronectin and \( \alpha_6 \beta_1 \) integrins (Schwartz and Denninghoff, 1994). Although fibronectin binding to these receptors is much weaker than binding to\( \alpha_v \beta_3 \), the former interaction evokes the rise in intracellular \( \text{Ca}^{2+} \) that was observed in cells that adhered to fibronectin.

Although tenasin-C does not mediate strong keratinocyte adhesion, it may well affect adhesion of keratinocytes to other proteins. It is known, for example, that tenasin-C inhibits fibronectin adhesion of various cells (Lotz et al, 1989; Lichter and Erickson, 1990; Murphy Ullrich et al, 1991; Prieto et al, 1993). Modulation of keratinocyte adhesion might be a major function of tenasin-C in vivo. This may be especially important during wound healing, where keratinocytes have to migrate to close the wound. Tenasin-C might facilitate keratinocyte migration by weakening the adhesion to fibronectin and possibly other extracellular matrix proteins that could otherwise interfere with keratinocyte migration and subsequent wound closure. Modulation of keratinocyte adhesion by tenasin-C is likely to be receptor-mediated. In the literature, various tenasin-C receptors have been described (Bourdon and Ruoslahti, 1989; Salmivirta et al, 1991; Prieto et al, 1993; Sriramarao et al, 1993; Barnea et al, 1994; Chung and Erickson, 1994; Vaughan et al, 1994; Yokosaki et al, 1994; Varnum Finney et al, 1995), and of these, the \( \alpha_v \beta_3 \) integrin is an interesting candidate.

Keratinocytes normally do not express \( \alpha_v \beta_3 \) but expression is induced in vivo after subculturing of keratinocytes and in vitro\( \alpha_v \beta_3 \) is expressed by wound keratinocytes (Breuss et al, 1995; Haapasalo et al, 1996). The site of \( \alpha_v \beta_3 \) expression, namely, the basal keratinocytes of the neo-epidermis, and the level of \( \alpha_v \beta_3 \) expression coincides with the site and level of tenasin-C expression. Receptors other than \( \alpha_v \beta_3 \) could also bring about adhesion modulation by tenasin-C, such as the \( \beta_1 \) integrins. Expression of \( \beta_1 \) integrins generally is strongly upregulated in wounds (Grinnell, 1992; Herfte et al, 1992; Larjava et al, 1993; Watt and Jones, 1993) and various \( \beta_1 \) integrins are able to bind tenasin-C (Bourdon and Ruoslahti, 1989; Prieto et al, 1993; Sriramarao et al, 1993; Yokosaki et al, 1994; Varnum Finney et al, 1995). Among the non-integrin-type tenasin-C receptors described, syndecan and annexin II could be important (Salmivirta et al, 1991; Chung and Erickson, 1994). The expression of the membrane-bound proteoglycan syndecan is altered during wound healing in the epidermis and the granulation tissue, the sites where we found changes in tenasin-C expression as well (Elenius et al, 1991; Oksala et al, 1995). Expression of the glycoprotein annexin II during wound healing has not been studied, but annexin II is present on the surface of keratinocytes in vitro and in vivo in normal and pathologic conditions (Culard et al, 1992; Bastian et al, 1993; Ma et al, 1994). Moreover, recent findings of Chung et al (1996) support the idea that annexin II could have a major role in tenasin-C-mediated effects on cell migration.

We conclude that keratinocytes are a major source of tenasin-C in the early phase of normal human wound healing, and we hypothesize that tenasin-C may have an anti-adhesive effect on keratinocytes.

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Figure 9. Keratinocyte morphology in adhesion assays. Values indicate the percentages of the attached feeder-cultured keratinocytes that have spread. Data were obtained from three or four separate experiments. Error bars, SEM [i.e., (LN) and fibronectin (FN), \( n = 12 \) (without PMA) and \( n = 5 \) (with PMA), respectively; tenasin-C (TN), \( n = 12 \) in both conditions].