Increased expression of tenascin C by keloids in vivo and in vitro

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Summary

Tenascin C, undulin, collagen XIV and fibronectin are extracellular matrix glycoproteins with a partial DNA sequence homology. During embryogenesis, tenascin C is abundant in mesenchymal tissues but its distribution in human adult tissue is severely restricted. The levels of tenascin C expression are enhanced with skin inflammation, wound healing and hyperproliferative skin diseases and return to normal in normal scar tissue after wound contraction is completed. Undulin/collagen XIV is associated with collagen fibrils and fibronectin is present throughout the dermis in adult skin but it is produced by keloidal fibroblasts in an increased amount. In this study we investigated by immunohistochemistry the expression of the three extracellular matrix proteins in keloids and normal skin as well as in keloidal and normal fibroblasts in vitro. In keloids, increased tenascin C expression was observed especially in the reticular dermis associated with collagen fibrils sharply demarcating the limit of the lesion. In normal tissue, tenascin C was only expressed beneath the basal lamina and dermo–epidermal junction. Corresponding to the in vivo findings, tenascin C expression was increased in keloidal fibroblasts compared with normal fibroblasts in vitro (P<0.003), whereas undulin/collagen XIV and fibronectin expression in keloids and keloidal fibroblasts was similar to that in normal tissue and normal fibroblasts, respectively. Therefore, tenascin C is a marker associated with keloids and we suggest that keloidal fibroblasts, once stimulated, continue to produce tenascin C independently from circulating factors.

Key words: collagen XIV, extracellular matrix glycoprotein, fibronectin, keloids, tenascin C, undulin

Keloids are characterized by abundant dense fibrotic tissue developing due to abnormal wound healing after skin injury. The mechanism of keloid formation is still unknown.1,2 Clinically, keloids extend beyond the confines of the original wound, do not regress spontaneously and tend to recur after excision.3,4 Histologically, they consist of excessive amounts of thick, densely packed eosinophilic collagen bundles in a focal or irregular pattern with only a few fibroblasts and often a mucoid glycosaminoglycan-rich matrix in between.4,5 Furthermore, appendages are missing, the epidermis seems to be thin and the papillary structures nearly disappear with effacement of the usually clear borders between the keloid and the reticular dermis.5,6 Keloids are rich in extracellular matrix components, especially fibrillar collagen,7–9 paralleled by elevated levels of fibrogenic cytokines, such as transforming growth factor (TGF)-β1.10 TGF-β1 has been shown to enhance the expression of several collagen types, fibronectin and tenascin C in cultured fibroblasts.10–12

In view of its expression in proliferating and activated extracellular matrices,13–16 tenascin C could be an interesting molecular marker associated with keloids. Tenascin C and the collagen fibril-associated new extracellular matrix protein undulin/collagen type XIV (C XIV), which is found in differentiated, quiescent matrices, exhibit complementary patterns of expression.17,18 As the expression patterns of tenascin C and C XIV in keloids are not known, we studied the immunohistochemical distribution and the expression by cultured fibroblasts of these molecules and compared them with those of fibronectin for which a strong expression in keloids has been shown.19

Materials and methods

Patients, cell cultures and methods

Biopsies from four untreated keloids (duration of disease 1–4 years) and four normal skin specimens comparable...
in sex (three men, one woman) and localization (earlobe, breast and back) were examined immunohistochemically. Keloids had developed after injury: ear piercing (two), surgical excision (one) and scarring acne (one). Primary fibroblast cultures were established from nine keloids (six men, three women; duration of disease 1–7 years) and five normal skin specimens (two men, three women) comparable in age (15–58 years) and localization (earlobe, breast, shoulder and back). Three of five normal skin specimens originated from patients with keloids included in the study. Tissue specimens were snap-frozen in liquid nitrogen and stored at \(-80^\circ C\) until use. Five-micrometre serial sections were placed on poly-L-lysine solution-coated slides, air-dried and stored in sealed boxes at \(-20^\circ C\) for no longer than 14 days. Before use, the sections were thawed slowly and fixed in acetone at \(4^\circ C\) for 10 min.

**Fibroblast cultures**

Primary fibroblast cultures from keloids and normal skin were grown from fresh dermal tissue specimens left to attach on culture dishes (Falcon, Franklin Lakes, NJ, U.S.A.) for about 2 weeks. Tissue and cell cultures were maintained in Dulbecco’s minimal essential medium (Seromed-Biochrom, Berlin, Germany) supplemented with 5% fetal calf serum and antibiotics. The cultures were subjected to immunocytological investigations in passages 3–4: \(2 \times 10^3\) cells were seeded in each well of modified eight-chamber culture vessels (Nunc, Wiesbaden, Germany) maintained in 300 µL culture medium and left to grow until subconflueny for 4 days at \(37^\circ C\) under 5% CO₂. The slides were then separated from the culture vessels, carefully washed with phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺, air-dried and stored in sealed boxes at \(-20^\circ C\) for up to 14 days. Before use, the slides were thawed slowly, fixed in 1% glutaraldehyde solution for 1 h and treated with 0·2% Triton X-100 solution for 10 min at room temperature.

**Immunohistochemical staining**

Alkaline phosphatase–antialkaline phosphatase staining was performed following established procedures. Monospecific rabbit antisera against human tenascin C (Biomol-Telios, Hamburg, Germany; dilution 1 : 50), human undulin (dilution 1 : 100) and human fibronectin (Dako, Hamburg, Germany; dilution 1 : 800) were diluted in RPMI medium (Gibco, Berlin, Germany). The primary antibodies were incubated with the samples for 30 min and the specimens were washed thoroughly in PBS. The sections were then incubated with alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Dianova, Hamburg, Germany) diluted 1 : 100 in a 1 : 1 mixture of human serum and RPMI medium for 30 min and washed as described before. The immunoreactants were visualized by incubation in a solution containing naphthol salt, hexazotized new fuchsin and levamisole, counterstained with haematoxylin and mounted in Kaiser’s gelatin solution according to standard procedures. Negative controls, prepared by the same procedure but omitting the primary antibodies, were included.

**Semiquantification of results**

The slides were viewed under a Leitz microscope. The intensity of staining in the tissue specimens was graded from \((-\) to \((+++)\) and its distribution was noted. For evaluation of the fibroblast cultures, all examined in subconfluent state, we compared the intensity of staining and used for statistical analysis five grades from 0 to 4 corresponding to negative, weak, average, strong and very strong staining. Statistical significance was calculated using Student’s \(t\)-test.

**Results**

**Tissue staining**

In all four samples of normal adult skin tenascin C was continuously expressed as a thin linear band associated with the basement membrane of the dermal–epidermal junction, being pronounced at the tips of the rete ridges. It was further found near the basement membrane of vessels, sebaceous glands and hair follicles (Fig. 1). There was no tenascin C staining in the dermal connective tissue, especially no distribution with the collagen fibres, and there was no difference in tenascin C staining between the papillary and reticular dermis. Tenascin C staining was markedly enhanced in all four keloid specimens examined. In contrast to normal skin, it was diffusely expressed throughout the dermis and often associated with the increased number of collagen bundles which characterize keloids (Fig. 2). There were more such collagen deposits with tenascin C colocalization in the reticular than in the papillary dermal layer. Here, tenascin C showed a wavy pattern along the collagen fibrils and was pronounced near the basement membrane, subepidermally in the flattened rete ridges.
and around the sparse vessels. There was a sharply demarcated border of tenascin C positivity with the surrounding unaffected dermal tissue (Fig. 3).

Unlike tenascin C, the distribution of C XIV in the dermis of keloidal and normal skin was similar. A strong staining was observed in association with the collagen fibres throughout the dermal connective tissue. In addition, there was a weak staining in the inner root sheath of the hair follicles in one of four normal skin specimens. In contrast to the restricted distribution of tenascin C in normal skin, fibronectin was very strongly expressed in a diffuse pattern throughout the dermis, especially along collagen fibres. The staining pattern and the distribution of fibronectin in dermal tissue were similar in normal skin and in all keloids examined. The epidermis remained negative. In addition, there was staining of the vessel walls in normal skin and in keloid lesions.

Expression in fibroblast cultures

The cytomorphology of keloidal and normal fibroblasts was similar. The staining of keloid-derived fibroblasts with tenascin C was strong to very strong in seven of nine cultures (Fig. 4), whereas the intensity of staining of normal fibroblasts for tenascin C (Fig. 5) was significantly weaker ($P<0.003$; Table 1). Pronounced immunoreactivity was located in secretory vesicles from keloidal and normal fibroblasts. There was no difference between the normal fibroblasts derived from patients with and without keloids (Table 1). In accordance with the findings on the tissue sections, C XIV was similarly expressed (average grade) in all keloidal and normal fibroblasts (Table 1). We could not find any difference between normal fibroblasts derived from patients with and without keloids either in intensity or in staining pattern. Keloidal and normal fibroblasts showed a nearly homogeneous distribution of C XIV in vesicles. Fibronectin displayed an average grade staining intensity in keloidal fibroblasts, resembling the intensity of normal fibroblasts (Table 1). There was no difference between the normal fibroblasts derived from the patients with and without keloids. Keloidal and normal fibroblasts showed a homogeneous distribution of fibronectin in the cell cytoplasm, whereas in some keloidal fibroblasts it was pronounced around the nucleus.

Discussion

Our immunohistochemical analysis showed a marked increase in the expression of tenascin C in keloids. It was diffusely distributed in the reticular and papillary dermis associated with collagen fibrils of affected tissue. In

Figure 1. In normal skin, tenascin C is expressed as a thin linear subepidermal band associated with the basement membrane of the dermal–epidermal junction, as well as in vessel walls and appendages (original magnification $\times 80$).

Figure 2. In a keloid, tenascin C is diffusely expressed throughout the lesion and appears to be associated with an increased number of collagen fibres (original magnification $\times 312.5$).
normal skin, tenascin C was restricted to the dermal–epidermal junction in the superficial papillary dermis and close to the basement membrane of vessels, ducts of glands and hair follicles as observed by others. In line with our in vivo results, keloidal fibroblasts in vitro exhibited significantly stronger tenascin C expression than normal fibroblasts. As the immunocytochemical studies were performed in passages 3–4, a major influence of stimulating systemic factors can be excluded. Therefore, we suggest that keloidal fibroblasts, once stimulated, autonomously produce tenascin C in abnormally high amounts. In contrast to the findings with tenascin C, we could not detect any differences either in intensity or in distribution of the staining pattern of C XIV or fibronectin between keloids and normal skin. The strong staining for C XIV was strictly associated with densely packed collagen fibres. In addition, no differences in intensity and staining pattern for both proteins were found between cultured keloidal and normal fibroblasts in vitro. In comparison with the marked dermal staining in vivo, the expression of C XIV in vitro seemed weaker. Our findings with fibronectin confirm previous results in vivo and in vitro. This suggests that there could be a different regulation of keloidal fibroblasts to produce abnormally high amounts of tenascin C continuously, in contrast to the synthesis of C XIV and fibronectin, although both fibronectin and tenascin C have been reported to be upregulated by TGF-β1, a fibrogenic cytokine reported to be increased in keloids.

Tenascin C is a large hexameric glycoprotein produced by mesenchymal cells such as fibroblasts or myocytes. The complete molecular sequence has been determined in humans and a partial homology between tenascin C, fibronectin and C XIV has been found. Tenascin C is strongly expressed in several organs during embryogenesis, being involved in epithelial–mesenchymal interactions, whereas in adult skin its expression and distribution become restricted. In normal skin tenascin C is located at the dermal–epidermal junction and it surrounds blood vessels and epidermal adnexa. Tenascin C expression increases in conditions characterized by wound repair and inflammation and malignant growth and was previously suggested as a marker for malignant transformation. However, it has also been found to be increased in benign lesions such as acne-induced keloids, scleroderma and hyperproliferative skin diseases. In vitro studies have shown that tenascin C modulates cell proliferation, migration and adhesion by interfering with fibronectin.

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**Table 1.** Immunocytological staining of keloidal and normal fibroblast cultures (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Keloidal fibroblasts</th>
<th>Normal fibroblasts</th>
<th>Normal fibroblasts of keloid patients</th>
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<tbody>
<tr>
<td>Tenascin C</td>
<td>3·4 ± 0·88</td>
<td>2·0 ± 0·0</td>
<td>2·0 ± 0·0</td>
</tr>
<tr>
<td>Undulin/collagen XIV</td>
<td>2·0 ± 0·0</td>
<td>2·0 ± 0·0</td>
<td>2·0 ± 0·0</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>2·0 ± 0·0</td>
<td>2·0 ± 0·0</td>
<td>2·0 ± 0·0</td>
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0, negative; 1, weak; 2, average; 3, strong; 4, very strong.

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action and by directly modulating cell differentiation and induction of differential gene expression. Tenascin C is upregulated in response to migrating keratinocytes but is also strongly expressed in granulation tissue and at wound edges in the dermis. After wound contraction is complete, tenascin C disappears more rapidly than other extracellular matrix proteins. The mechanism of tenascin C induction in keloids is still unknown. Previous studies reported that TGF-β1 and interleukin 1β are potent inducers of tenascin C. Interestingly, TGF-β1 is increased in keloids and also induces fibronectin and collagen expression. Shah et al. demonstrated that wounds in adult rats healed without scar formation after treatment with neutralizing antibodies to TGF-β. Although many mesenchymal cell types are able to express tenascin, dermal fibroblasts are assumed to be the main source of this protein in keloids, the expression of which may reflect the ongoing fibrogenic process. It is not known whether keloidal fibroblasts require a stimulating serum factor or whether they undergo further stimulation in an autocrine/paracrine manner. Our findings on cultured keloidal fibroblasts suggest that keloidal fibroblasts do not require systemic stimulation to maintain their upregulated tenascin expression. On the other hand, we found in vitro that keloidal fibroblasts significantly decrease their tenascin C expression after cryotherapy, in contrast to normal fibroblasts which increase their tenascin C production (data not shown). Therefore, we assume that keloidal fibroblasts are characterized by a defective feedback mechanism regulating the production of tenascin C, possibly correlated with a protracted fibrogenic activation.

Recent work showed the identity of undulin with C XIV, a large non-fibrillar extracellular matrix protein that belongs to the subfamily of fibril-associated collagens. Its DNA sequence reveals 42% homology with regions of human fibronectin and 33% homology with parts of tenascin C. Despite their molecular similarities, C XIV plays a divergent biological role compared with tenascin C and fibronectin. It is abundant in flexible connective tissues that contain large amounts of collagen fibrils. In scar tissue, C XIV expression is rare and it becomes downregulated more rapidly than any other extracellular matrix protein. C XIV was suggested therefore to play a part in the supramolecular organization of collagen fibrils to fibres leading to flexibility and the stability characteristic for differentiated mesenchymal tissues. Although several mesenchymal cell lines produce C XIV, extremely low levels were found in human skin fibroblasts. In the dermis, C XIV is found on densely packed collagen fibres and next to vessels and appendages. Prominent expression of C XIV has also been described around growing hair follicles. Our findings partially confirm these previous results: C XIV was associated with collagen fibrils and, in addition, a weak C XIV staining was present next to hair follicles in normal dermis, but not around capillary blood vessels nor around glands.

Fibronectin, a well-known non-collagenous glycoprotein, is abundant in most interstitial connective tissues. Fibronectin is also located on collagen fibrils where it may mediate the interaction between cells and other matrix components such as collagens. It plays an important part in wound healing, promoting the development of granulation tissue. There are reports of both increased and normal fibronectin synthesis in keloidal fibroblasts.
In summary, our data show that despite some molecular similarities, tenascin C, C XIV and fibronectin display characteristic and distinct distribution patterns in normal skin in correlation with their suggested functions in mesenchymal tissue. Whereas C XIV and fibronectin preserve their distribution pattern in keloids, the pattern of tenascin C changes significantly. In keloids it is expressed de novo throughout the dermis, mostly in proximity to collagen fibres. Thus, tenascin C is an excellent marker associated with keloids, demarcating the border with unaffected skin. In addition, keloidal fibroblasts produce higher amounts of tenascin C in vitro than normal fibroblasts without stimulation by external factors, a finding that supports an altered feedback mechanism for tenascin C expression in keloidal fibroblasts.

Acknowledgments

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References