Fibroblasts from post-burn hypertrophic scar tissue synthesize less decorin than normal dermal fibroblasts

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INTRODUCTION

Hypertrophic scarring (HSc) commonly occurs in healing deep second-degree burns. The scars are red, raised, itchy and inelastic and may impair the mobility of underlying joints. Resolution is usually spontaneous but may take several years. The undesirable physical properties of HSc are a direct consequence of the deranged organization of the dermis in which the collagen fibrils are narrower, more widely spaced and often arranged in whorls or nodules rather than the fibres and fibre bundles running parallel to the surface seen in normal skin or mature scar [1]. Disorganization of the collagen in turn may result from abnormalities in type and/or amount of proteoglycans (PGs) and glycoproteins. Hypertrophic scar is known to contain more water than normal dermis or mature scar [2], higher concentrations of the glycosaminoglycan components uronic acid and hexosamine [3] and more fibronectin [4]. The glycosaminoglycans differ qualitatively from those in normal dermis: chondroitin sulphate (CS) which is normally barely detectable is readily demonstrated in HSc [5] and dermatan sulphate (DS), the major glycosaminoglycan of normal dermis, is virtually absent [6]. Dermatan sulphate in skin is almost entirely present as the single glycosaminoglycan chain on the PG DS-PGII/decorin [7]. Cultured fibroblasts also produce DS-PGII/biglycan but this is a minor PG in most adult connective tissues except articular cartilage [8] and fibrocartilage [9-11]. A large CS-containing PG (versican) is found in small amounts in skin [12]. Our own recent immunohistochemical [13] and chemical [14] studies showed the presence of abnormally high levels of biglycan and versican in HSc and the virtual absence of decorin. The levels of these PGs were normal in mature post-burn scars. The present investigation was undertaken to determine whether these characteristics of HSc tissue could result from intrinsically different biosynthetic activity of the resident fibroblasts. The effects of transforming growth factor-β1 (TGF-β1) on these cells were also studied since this cytokine is the one most often thought to be involved in the pathogenesis of fibrotic conditions such as HSc [15].

1. Fibroblast cultures were established from biopsies of hypertrophic scar and normal dermis taken from nine patients recovering from second- and third-degree burns. The capacity of these fibroblasts to synthesize the small proteoglycan decorin was assessed by quantitative Western blot analysis of conditioned medium collected from confluent cultures. Levels of mRNA for decorin were assessed by quantitative Northern analysis. Since transforming growth factor-β1 is implicated in various fibrotic conditions, including post-burn hypertrophic scar, its effect on decorin synthesis by these paired fibroblast cell strains was assessed.

2. Production of decorin was lower in all cell strains of hypertrophic scar fibroblasts tested, compared with normal dermal fibroblasts cultured from the same patients (mean 49 ± 23%; P < 0.001, n = 9). Levels of mRNA for decorin were also lower (mean 59 ± 28%; P < 0.02, n = 7) but those for biglycan and versican were not significantly different. Four pairs of cell strains were examined at more than one passage and the differences in decorin protein were found to be phenotypically persistent. Treatment of confluent cultures with transforming growth factor-β1 for 3 days caused a reduction in both decorin protein and mRNA in all six strains of normal dermal fibroblasts. An increased depression of decorin synthesis by transforming growth factor-β1 was reversed on removal of the agent and passing the fibroblasts.

3. The reduced capacity of fibroblasts in hypertrophic scar tissue to synthesize decorin may have implications for the development of the condition since this small proteoglycan is involved in tissue organization and may also play a role in modulating the activity in vivo of fibrogenic cytokines such as transforming growth factor-β1.

MATERIALS AND METHODS

HSc and control fibroblasts

Patients who had suffered thermal injuries and been treated at the University of Alberta Hospitals Fire-
Table 1  Sources of fibroblasts

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Biopsy site</th>
<th>TBSA (%)</th>
<th>Months post-burn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>M</td>
<td>Hand</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>F</td>
<td>Arm</td>
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<td>14</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>M</td>
<td>Face</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>M</td>
<td>Chest</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>M</td>
<td>Thigh</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>M</td>
<td>Chin</td>
<td>75</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>M</td>
<td>Chest</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>M</td>
<td>Neck</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>M</td>
<td>Leg</td>
<td>11</td>
<td>27</td>
</tr>
</tbody>
</table>

*Percentage of total body surface area affected.

Western blot immunoassay of decorin secreted by cultured fibroblasts

Medium in five 75-cm² flasks of confluent fibroblasts (1–2 × 10⁶ cells/flask) was replaced with 7 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and allowed to reach confluence.

The cDNA probes

The cDNA probes for human decorin and versican were obtained from Telios Pharmaceuticals (San Diego, CA, U.S.A.). The probe for decorin was an amplified polymerase chain reaction product corresponding to bp 727–1348 of human decorin cDNA [21]. The versican probe was a polymerase chain reaction product corresponding to bp 1475–2802 of versican cDNA [22]. The probe for human biglycan was a fragment of 1685 bp kindly provided by Dr. L. W. Fisher (Bone Research Branch, NIDR, NIH, Bethesda, MD, U.S.A.) [23]. The cDNAs for 18S rRNA and /-actin mRNA were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Probes were labelled with [α-³²P]dCTP (DuPont NEN, Boston, MA, U.S.A.) by nick-translation.

Extraction and hybridization of mRNA

Culture medium was removed from four 75-cm² flasks of confluent fibroblasts. Cell layers were lysed in 6 ml of guanidinium isothiocyanate and RNA was extracted using the guanidinium isothiocyanate/CsCl procedure of Chirgwin et al. [24]. Total RNA was separated by electrophoresis and blotted onto nitrocellulose membranes which were then baked under vacuum for 2 h at 80 °C and prehybridized for 4 to 6 h at 42 °C. These procedures and the hybridization with cDNA probes and subsequent washings and autoradiography were carried out as described previously [25].

Quantitative analysis of Western and Northern blots

Autoradiograms were scanned using a Macintosh Color OneScanner™ and the data imported into the program NIH Image. Each Western blot included known amounts of bovine skin decorin which were used to construct a standard curve to which the unknowns, run in duplicate, were compared. If preliminary analysis gave a measured density for an unknown outside the range of the standards, that sample was re-run at a different loading. Results were
Decorin synthesis by burn scar fibroblasts

then corrected for the number of cells in the flask from which medium had been collected. Ratios of the densities of proteoglycan mRNA to 18S rRNA or β-actin mRNA on Northern blots were calculated to correct for variations in gel loading. Visual inspection of ethidium bromide-stained gels confirmed that levels of 18S rRNA or β-actin mRNA did not vary significantly between HSc and normal fibroblasts or as a result of treatment with TGF-β1. The statistical significance of differences in decorin protein production and mRNA levels between normal and HSc fibroblasts and between TGF-β1-treated and untreated fibroblasts was tested using Wilcoxon’s signed rank test. P-values less than 0.05 were considered significant.

RESULTS

Secretion of decorin into cell culture medium

Figure 1 shows the results of a typical chemiluminescent Western blot in which decorin secreted by two pairs of HSc and normal dermal fibroblasts is compared, together with standards of pure decorin from bovine skin. The HSc cells can be seen to produce a smaller amount of decorin with a lower electrophoretic mobility than the normal dermal fibroblasts from the same patients. The lower mobility is due to the presence of a longer DS chain on decorin from the HSc fibroblasts, as demonstrated by digestion with chondroitinase ABC which generated protein cores of identical size (apparent molecular mass 43–45000 Da) (Figure 2). The absolute amounts of decorin produced by the different strains of fibroblasts varied widely but in each case cells from the scar produced less decorin (1673 ± 1405 ng/10^6 cells, mean ± S.D.) than the normal dermal fibroblasts from the same patient (3620 ± 3303 ng/10^6 cells). Production of decorin by cells from HSc was 49 ± 23% (mean ± S.D.) of that produced by normal dermal fibroblasts. This difference was highly significant (P < 0.001, n = 9) by the Wilcoxon signed rank test. Decorin production by four of the pairs of cell strains (those from patients 3, 4, 6 and 9) was examined at two passages (6 and 8, 3 and 4, 6 and 7 and 3 and 4 respectively) and in each case the relatively lower decorin production by the HSc fibroblasts persisted at the higher passage number.

Expression of decorin mRNA

As shown in Figure 3, decorin cDNA hybridized to two transcripts with apparent sizes of 1.6 and 1.9 kb, consistent with previous reports [26]. In all seven HSc

![Figure 1](image1.png)

**Figure 1** Comparison of decorin protein secreted by fibroblasts from hypertrophic scar (H) and normal dermis (N) from two patients (7 and 9 in Table 1)

Each sample was run in duplicate on a 7% polyacrylamide gel, transferred to nitrocellulose and stained using the monoclonal antibody 6D6 and a chemiluminescent detection system. The five lanes to the right carry known amounts (250–4500 ng) of purified bovine skin decorin (BS-DCN), used to construct a standard curve for quantification of decorin in cell culture supernatants. See text for other details.

![Figure 2](image2.png)

**Figure 2** Comparison of electrophoretic mobilities of intact decorin and protein cores synthesized by normal (N) and hypertrophic scar (H) fibroblasts, without and with (lanes marked +) treatment with TGF-β1 for 72 h

The right lane in each pair carries the protein core produced by digestion of the material in the left lane with chondroitinase ABC, as described previously [11]. Other details as for Figure 1.

![Figure 3](image3.png)

**Figure 3** Comparison of levels of decorin mRNA in fibroblasts from hypertrophic scar (H) and normal dermis (N) from two patients (2 and 3 in Table 1)

The right lane of each pair (marked +) carries RNA from cells treated with TGF-β1 for 72 h. The blot was probed for decorin mRNA (DCN), stripped and then reprobed for 18S rRNA. The two autoradiograms were superimposed for photography.
fibroblast strains tested there was less mRNA for decorin than in the normal fibroblasts from the same patient. Densitometric determination of the ratio of decorin mRNA to 18 S rRNA showed that decorin mRNA was significantly less abundant in HSc fibroblasts (59 ± 28%, mean ± S.D.; n = 7, P < 0.02).

There was considerable variability in levels of versican and biglycan mRNA between cell strain pairs but no consistent differences between HSc and normal dermal fibroblasts from the same patient (Figure 4). Slightly lower average levels of these mRNAs (96% for versican and 91% for biglycan) were found in the seven HSc cell strains tested than in the normal dermal fibroblasts but these small reductions were not statistically significant.

**Effects of TGF-β1 on decorin secretion and mRNA levels**

Treatment with TGF-β1 for 3 days reduced the levels of decorin protein production and decorin mRNA in all six strains of HSc fibroblasts and in five of the six strains of normal dermal fibroblasts tested. The reductions in both protein (35.5%) and mRNA (40.2%) were significant for the HSc fibroblasts (P < 0.05) while that for mRNA in the normal fibroblasts (33.0%) was significant (P < 0.05) and for protein (20.1%) marginally significant (P = 0.06). The HSc fibroblasts appeared to be somewhat more sensitive to the effects of TGF-β1 than the normal dermal fibroblasts but this difference in response was not statistically significant. In four of the six cell strain pairs tested there was a visible increase in the size of the decorin as judged by its mobility on SDS gel electrophoresis (Figure 5). Examination of the protein cores produced by digestion of the PGs with chondroitinase ABC showed that this increase in size was due to the DS chain (Figure 2). To test whether the effect of TGF-β1 on decorin production by these fibroblasts was phenotypically persistent, one pair of cell strains (those from patient #4) was treated for 3 days at passage 4 and then allowed to recover in Dulbecco's modified Eagle's medium with 10% fetal bovine serum without added TGF-β1 for 4 days. At that time decorin protein production had returned to 83% of the untreated level in the HSc fibroblasts and to 84% in the normal dermal fibroblasts (compared with 40.7 and 67.7% respectively immediately after treatment with TGF-β1). Recoveries of decorin mRNA levels paralleled those for protein production. After two passages in culture, during which the cells were split 5-fold each time after trypsinization, decorin production rose to 117% and 109% of the untreated levels in the HSc and normal dermal fibroblasts respectively.

**DISCUSSION**

When comparisons are made on a patient by patient basis, the deficiency in capacity of fibroblasts from HSc to synthesize decorin, compared with those from normal dermis, is significant. This may be ascribed at least in part to lower steady-state levels of decorin mRNA which are strongly correlated with protein production (by linear regression analysis r² = 0.75, P < 0.0001, n = 14; results not shown). Wide variation was seen between fibroblast strains from different patients in both decorin production and mRNA levels, such that in unpaired comparisons this variability could obscure the differences. The paired study design, which we also used in an earlier investigation [27], should reduce or eliminate variability in the behaviour of fibroblasts due to factors such as the age, sex and general state of health of the donor and, perhaps most importantly, the explantation process itself. For these experiments biopsies of scar and normal skin were obtained from the same patient at the same time and treated identically throughout the study.

The reduced capacity of the cultured HSc fibroblasts to synthesize decorin appears to reflect the activity of these cells in the tissue from which they were derived, where we recently reported the concentration of this PG to be only about 25% of that in normal dermis or in post-burn mature scars [14]. A qualitative observation which further supports the assertion that the behaviour of the fibroblasts in vitro at least partially...
reflects that in vivo, is the finding that decorin synthesized by HSC fibroblasts migrates more slowly on SDS gels than that from normal fibroblasts. This previously reported characteristic of decorin isolated from burn scars [14,28] is conferred on it by the DS chain (which is probably longer) since the protein cores are indistinguishable ([14] and present results). Although a reduced degree of sulphation could theoretically have the same effect on the electrophoretic mobility as a longer glycosaminoglycan chain, it seems likely that SDS in the gel would mask any minor differences.

Interestingly, the levels of the mRNAs for versican and biglycan, two PGs which are present in much higher concentrations in HSC tissue than in normal dermis or mature scar [14], were not significantly altered in the fibroblasts studied here. Consequently we did not consider it worthwhile to measure levels of secreted versican and biglycan proteins in the present study. Presumably the enhanced production of these two PGs in HSC is stimulated by factors present in the healing wound but absent from the culture system.

In addition to the decreased ability to synthesize decorin demonstrated here, fibroblasts from HSC have been shown by us and others to synthesize less nitric oxide [29], much less collagenase [30] and to divide rather more slowly than normal dermal fibroblasts [15,31]. The altered phenotype of HSC fibroblasts presumably results from selective expansion of certain populations of cells within the environment of the healing wound. A simple explanation might be that many of these fibroblasts are derived from deep dermal elements which survive the tissue damage associated with second-degree burns. Fibroblasts from the reticular dermis have been reported to contain lower levels of decorin mRNA and to synthesize less decorin than those from the papillary dermis [32]. Other explanations could involve altered and/or elevated levels of cytokines in the wound.

TGF-β1, the cytokine most strongly implicated in fibroproliferative disorders [15], has a number of cellular effects which could contribute to the development of these conditions. It stimulates the synthesis of collagen and fibronectin by normal fibroblasts [33] and alters patterns of synthesis of PGs by increasing levels of mRNA for versican and biglycan and decreasing that for decorin [34]. It suppresses the induction of collagenase in quiescent cells by epidermal growth factor and basic fibroblast growth factor [35] and suppresses the activity of TGF-β1 [36]. It has also been reported to play a role in the organization of collagen fibrils into the suprafibrillar structures: fibres and fibre bundles [37].

It is possible that the reduced synthesis of decorin by HSC fibroblasts in vitro is a consequence of the elevated production of TGF-β1, which we have noted for these same cells (P. G. Scott and C. M. Dodd, unpublished work). However, if this were true we would have expected to find increased levels of mRNAs for versican and biglycan [34] and this was not the case. Clearly further work will be required to define the alteration in the HSC fibroblasts that is responsible for their reduced capacity to synthesize decorin.

In the present investigation we found that fibroblasts cultured from either normal dermis or HSC in the presence of TGF-β1 had lower steady-state levels of decorin mRNA and synthesized less decorin protein. Earlier reports on the effects of TGF-β1 on synthesis of PGs by fibroblasts were conflicting: Bassols and Massague [39] reported an increase in DS–PGI production but did not distinguish decorin (DS–PGI) from biglycan (DS–PGII); Westergren-Thorsson et al. [40] found increased synthesis of both decorin and biglycan, although more pronounced for the latter, and Kahari et al. [34] reported that levels of versican and biglycan mRNAs were increased but those for decorin decreased. The present results on decorin mRNA are in accord with those of Kahari et al. [34] and with the more recent identification of a TGF-β1-negative regulatory element in the human decorin gene promoter [41]. None of these earlier studies tested fibroblasts from HSC, which we found here to be possibly even more sensitive to TGF-β1 than those from normal dermis. Neither do there appear to be previous reports of the recovery of fibroblasts from the effects of TGF-β1.

During the healing of incisional wounds in humans and punch biopsy wounds in guinea pigs, strong immunohistochemical staining for decorin, indistinguishable from that in normal dermis, can be demonstrated in tissue sections after digestion with chondroitinase ABC to remove glycosaminoglycans masking the protein epitopes [42]. In post-burn HSC tissue, treatment with chondroitinase ABC has no effect on the intensity of staining for decorin [13] and the much lower concentration of this PG has been confirmed by extraction and analysis [14]. The observations of Yeo et al. [42] imply that early and abundant expression of decorin is a feature of normal wound healing. Delayed appearance of decorin may have a number of undesirable consequences for the development and properties of scar extracellular matrix. This PG is believed to play a role in the organization of collagen fibrils into the suprafibrillar structures: fibres and fibre bundles [43]. These structures, which are mainly oriented parallel to the tissue surface in normal dermis and give the tissue its isotropic distensibility, are absent from the nodular areas of hypertrophic scar [1]. Decorin has been reported to neutralize the activity of TGF-β1 in vitro [44] and in vivo [45,46] and immunohistochemical staining for TGF-β is much stronger in mature post-burn scars (which also contain normal levels of decorin) than in hypertrophic scars [13]. These observations suggest that decorin may be involved in the eventual spontaneous resolution of this debilitating condition.
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REFERENCES


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