Neutralisation of TGF-β₁ and TGF-β₂ or exogenous addition of TGF-β₃ to cutaneous rat wounds reduces scarring

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SUMMARY

Exogenous addition of neutralising antibody to transforming growth factor-β₁ to cutaneous wounds in adult rodents reduces scarring. Three isoforms of transforming growth factor-β (1, 2 and 3) have been identified in mammals. We investigated the isoform/isoforms of TGF-β responsible for cutaneous scaring by: (i) reducing specific endogenous TGF-β isoforms by exogenous injection of isoform specific neutralising antibodies; and (ii) increasing the level of specific TGF-β isoforms by exogenous infiltration into the wound margins. Exogenous addition of neutralising antibody to TGF-β₁ plus neutralising antibody to TGF-β₂ reduced the monocyte and macrophage profile, neovascularisation, fibronectin, collagen III and collagen I deposition in the early stages of wound healing compared to control wounds. Treatment with neutralising antibodies to TGF-β₁ and TGF-β₂ markedly improved the architecture of the neodermis to resemble that of normal dermis and reduced scarring while the control wounds healed with scar formation. Exogenous addition of neutralising antibody to TGF-β₁ alone also reduced the monocyte and macrophage profile, fibronectin, collagen III and collagen I deposition compared to control wounds. However, treatment with neutralising antibody to TGF-β₁ alone only marginally reduced scarring. By contrast, wounds treated with neutralising antibody to TGF-β₂ alone did not differ from control wounds. Interestingly, exogenous addition of the TGF-β₃ peptide also reduced the monocyte and macrophage profile, fibronectin, collagen I and collagen III deposition in the early stages of wound healing and markedly improved the architecture of the neodermis and reduced scarring. By contrast, wounds treated with either TGF-β₁ or with TGF-β₂ had more extracellular matrix deposition in the early stages of wound healing but did not differ from control wounds in the final quality of scarring.

This study clearly demonstrates isoform specific differences in the role of TGF-β isoforms in wound healing and cutaneous scarring. TGF-β₁ and TGF-β₂ are implicated in cutaneous scarring. This study also suggests a novel therapeutic use of exogenous recombinant, TGF-β₃ as an anti-scarring agent.

Key words: TGF-β₃, scarring, wound healing

INTRODUCTION

The transforming growth factor-β family (TGF-β) is a multifunctional regulator of cell-growth, differentiation and extracellular matrix formation (Roberts and Sporn, 1990). Three TGF-β isoforms are present in mammals. TGF-β₁, TGF-β₂ and TGF-β₃ genes are highly conserved between species. There is 64%-85% amino acid sequence homology between the three isoforms. In most in vitro assays, the biological activity of the three isoforms is similar (Graycar et al., 1989). However, there are differences in their potencies and some biological activities. TGF-β₁ is a 100-fold more potent growth inhibitor of haemopoietic stem cells than TGF-β₂; TGF-β₂ and TGF-β₃ but not TGF-β₁ induce mesoderm formation in early frog embryos; TGF-β₁ is more potent than TGF-β₁ and TGF-β₂ at inhibiting DNA synthesis in primary human keratinocyte cell cultures and TGF-β₁ and TGF-β₂ are more potent inhibitors of endothelial cell proliferation than TGF-β₂ (Graycar et al., 1989; Jennings et al., 1988; Roberts et al., 1990; Merwin et al., 1991).

Recent immunolocalisation and in situ hybridisation studies in developing mouse and human embryos have shown complex and distinct patterns of distribution of the three TGF-β isoforms. These studies suggest differential regulation of the three isoforms of TGF-β suggesting that they play unique roles during development (Pelton et al., 1990, 1991; Gatherer et al., 1990; Millan et al., 1991; Schmid et al., 1991).

Wound healing and embryogenesis have many similarities which include cell migration, proliferation, differentiation and extracellular matrix formation. Whilst most fetal tissues respond to injury by a process of regeneration, injury to most adult tissues terminates in scar formation (Whitby and Ferguson, 1991). We have shown that early application of neutralising antibody to TGF-β to wounds reduces scar formation (Shah et al., 1992, 1994). TGF-β₁ has been implicated in certain fibrotic disorders such as pulmonary fibrosis, glomerulonephritis, and cirrhosis of the liver (Border et al., 1990; Broekelmann et al., 1991; Castilla et al., 1991). TGF-β₂ has been implicated in proliferative vitreoretinopathy (Connor et
These pathological responses to injury may be due to an aberration in the regulation of the TGF-β isoforms. Therefore, the aim of the present study was to identify the isoform/isoforms of TGF-β responsible for scar formation after cutaneous injury in the adult rodent. The strategies used were: (i) to reduce specific endogenous TGF-β isoforms in the healing wound, by exogenous addition of isoform specific neutralising antibody; and (ii) to increase the level of a specific TGF-β isoform in the healing wound by exogenous addition of the TGF-β isoform. This study implicates TGF-β1 and TGF-β2 in cutaneous scarring whilst TGF-β3 reduces cutaneous scarring.

MATERIALS AND METHODS

Experimental model

We used the previously described experimental model for this study (Shah et al., 1994). Briefly, four full-thickness, linear incisions, 1 cm in length, down to and including the panniculus carnosus muscle, were made on the dorsal skin of adult, male Sprague-Dawley rats weighing 225-250 g, under halothane, oxygen and nitrous oxide anaesthesia. One of the wounds (control) was unmanipulated; the second wound (sham-control) was injected with PBS/0.1% BSA/4 mM HCl (carrier used to make up the TGF-β isoforms); the third wound was injected with an isoform specific neutralising antibody to TGF-β and the fourth wound was injected with the corresponding isoform of TGF-β (Shah et al., 1994). The actual site of each treatment was rotated between the four wounds to control for anterior-posterior differences in the healing of rodent wounds (Auerbach and Auerbach, 1982). All injections were of 100 µl each and administered by local infiltration into the dermis of the wound margins. The wounds were treated on days 0, 1 and 2 post-wounding. Animals were allowed to recover and housed in individual cages and fed normal rat chow and water ad libitum. Animals were killed by chloroform overdose on days 3, 5, 7 and 70 post wounding whilst wounds from group IV were harvested 14 days post-wounding.

Modulation of TGF-β isoform profile

To study the effects of manipulation of the TGF-β isoform profile of healing wounds, animals were divided into four experimental groups and wounds were treated as shown in Table 2 on days 0, 1 and 2 post-wounding. The neutralising antibodies to TGF-β1 and TGF-β2 used in these experiments were from British Biotechnology Ltd, Oxford and the neutralising antibody to TGF-β3 was from Genzyme Corp., Cambridge, USA (#1835-001; monoclonal mouse anti-bovine natural TGF-β2; 20-30 µg/ml IgG neutralises 0.10-0.50 ng/ml TGF-β1,2,3 in vitro). The control wounds were unmanipulated and the sham control wounds were treated with PBS/0.1% BSA/4 mM HCl.

The wounds from experimental groups I, II and III were harvested on days 3, 5, 7 and 70 post wounding whilst wounds from group IV were harvested on days 7 and 70 post-wounding. Four animals per time point per group were studied and 224 wounds were analysed in these experiments.

Analyses of wounds

After harvesting, the wounds were bisected, one half was frozen and processed for immunohistochemistry and the other half was fixed in 4% paraformaldehyde and processed for paraffin embedding.

Immunohistochemistry

The wounds were embedded in OCT compound (Miles Inc., Elkhart.

| Table 1. Doses of neutralising antibodies to TGF-β and TGF-β isoforms |
|-----------------|-----------------|-----------------|-----------------|
| Set             | Anti-TGF-β1 (µl/inj) | TGF-β1 (ng/inj) | Anti-TGF-β2 (µl/inj) | TGF-β2 (ng/inj) | Anti-TGF-β1 + anti-TGF-β2 (µl/inj, of each) | TGF-β3 (ng/inj) |
| A               | 0.01            | 0.1             | 0.01            | 0.1             | 0.01            | 0.1             |
| B               | 0.1             | 1.0             | 0.1             | 1.0             | 0.1             | 1.0             |
| C               | 1.0             | 50.0            | 1.0             | 10.0            | 1.0             | 10.0            |
| D               | 5.0             | 50.0            | 5.0             | 50.0            | 5.0             | 50.0            |

Neutralising antibody to TGF-β1 was raised in the turkey against native porcine TGF-β1; 1 µl of anti-TGF-β1 neutralises approximately 4 ng of TGF-β1 in vitro. Neutralising antibody to TGF-β2 was raised in the rabbit against native porcine TGF-β2; 1 µl of anti-TGF-β2 neutralises approximately 6 ng of TGF-β2 in vitro. TGF-β1 and TGF-β2 were extracted from porcine platelets; TGF-β3 was recombinant TGF-β3 expressed in NIH 3T3 cells.
These defined areas measured 0.162 mm$^2$ in area and were selected subepidermal and deep dermal regions of the wound were counted. monocytes and macrophages present in defined areas within the image analyser (Joyce Loeble Ltd., Gateshead, UK), the number of avidin. With the aid of epifluorescence microscopy and a Magiscan vidin. The stained sections were visualised by epifluorescence microscopy coated slides and stored at $-20^\circ$C until they were used for staining. The stained sections were visualised by epifluorescence microscopy using a Leitz Dialux microscope. Photography used identical settings for black and white photos or Kodak 200 ASA film for colour photography.

At least three sections from each wound harvested on days 3 and 7 post-wounding were immunostained for monocytes and macrophages using monoclonal antibody ED1 (Serotec Ltd, Oxford, UK), biotinylated secondary antibody and FITC-conjugated streptavidin. With the aid of epifluorescence microscopy and a Magiscan image analyser (Joyce Loeble Ltd., Gateshead, UK), the number of monocytes and macrophages present in defined areas within the subepidermal and deep dermal regions of the wound were counted. These defined areas measured 0.162 mm$^2$ in area and were selected such that they did not transgress the wound margins and were from the midline of the wounds. The selected area in the subepidermal region was immediately subjacent to the epidermis and that in the deep dermis was immediately above the level of the panniculus carnosus muscle (Shah et al., 1994).

Sections from 5 and 7 day wounds were immunostained for laminin (Chemicon Int. Inc., Temecula, USA) and von Willebrand factor (Dakopatts. Glostrup, Denmark) and the angiogenic response was scored. Sections from 7 day wounds were immunostained for fibronectin (Chemicon Int. Inc., Temecula, USA) and the intensity of staining was scored. Sections from 7 and 70 day wounds were immunostained for collagen I and collagen III (Chemicon Int. Inc., Temecula, USA) and the intensity of staining was scored. Appropriate FITC-conjugated secondary antibodies were used.

Routine histology
Paraffin sections (7 µm) of 70 day wounds were stained with Picrosirius Red. Using polarising microscopy, the architecture of the neodermis was compared with that of the normal dermis. Orientation of the collagen bundles, spacing between the bundles i.e. compactness and the size of the collagen bundles were taken into account for scoring.

For each parameter studied, comparison between treatments and controls was performed on wounds from the same animal to correct for interanimal variation. Sampling of the wounds to study each parameter was standardised by selecting at least 3 sections from identical sites within the wounds. Details of the staining techniques, antibodies used for immunostaining and the scoring systems are described elsewhere (Shah et al., 1994). All sections were scored by two independent observers.

Scoring system
A number of different scoring systems were investigated to evolve the one described herein. A key feature is that the same scoring system was used for all semiquantitative analyses, even though the potential negative scores were never achieved in some staining regimes. Further

<table>
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<td>Anti-TGF-β1 (50 µg/inj)</td>
<td>Anti-TGF-β2 (50 µg/inj)</td>
<td>Anti-TGF-β1 + anti-TGF-β2 (50 µg each/inj)</td>
<td>Anti-TGF-β1+2,3 (50 µg/inj)</td>
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<tr>
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<td>TGF-β2 (20 ng/inj)</td>
<td>TGF-β1 (20 ng/inj)</td>
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Animals were grouped into four experimental groups and treated as shown. Control wounds were unmanipulated; sham control wounds received PBS/0.1% BSA/4 mM HCl.

IN) and frozen in liquid nitrogen. Serial sections, 7 µm in thickness, were cut onto poly-L-lysine (Sigma Chemical Co., Dorset, UK) coated slides and stored at $-20^\circ$C until they were used for staining. The stained sections were visualised by epifluorescence microscopy using a Leitz Dialux microscope. Photography used identical settings and either T-Max 100 film for black and white photos or Kodak 200 ASA film for colour photography.

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RESULTS
In all experiments, there were no differences between the control and sham control wounds.

Dose response
The doses of the isofrom specific antibodies used in the first experiment (gift from Dr A. B. Roberts, Laboratory of Chemoprevention, Bethesda, USA) are expressed as µl/injection of the neutralising antiserum. The actual volume injected into each wound was 100 µl/injection (50 µl into each wound margin) made up by adding PBS.

Fibronectin deposition
Compared to the controls, wounds treated with 1 µl or 5 µl/injection of neutralising antibody to TGF-β1 (anti-TGFβ1) plus neutralising antibody to TGF-β2 (anti-TGFβ2) showed a lower intensity of staining for fibronectin. This reduction was more marked in wounds treated with 5 µl/injection of these antibodies. Wounds treated with 5 µl/injection of either anti-TGFβ1 or anti-TGFβ2 also showed some reduction in the intensity of staining for fibronectin. Wounds treated with the lower doses of all neutralising antibodies did not differ from control wounds. These effects were seen on both days 7 and 14 post-wounding (Fig. 1).

The intensity of staining for fibronectin in wounds treated with either 10 ng/injection or 50 ng/injection of TGF-β3 was less than that in control wounds. This effect was more marked in the deep dermal region of the wounds. By contrast, wounds treated with either 10 ng/injection or 50 ng/injection of TGF-β1 showed increased intensity of staining for fibronectin compared to that in control wounds. Wounds treated with 50 ng/injection TGF-β2 showed a marginal increase in fibronectin but, only in the subepidermal region of the wounds. These differences were also seen on day 14 post-wounding. (Fig. 2).

Angiogenic response
The angiogenic response was investigated using laminin and von Willebrand factor staining to mark the blood vessels of 7 day wounds. Wounds treated with 5 µl/injection of either anti-TGFβ1 or anti-TGFβ1 plus anti-TGFβ2 were less vascular than

TGF-β3 reduces scarring

The quantitative data of monocyte and macrophage profiles were subjected to repeated measurements by multivariate analysis of variance using SPSS programme. This analysis was used to compare differences between treatments and to study the interactions between time, treatment and depth (subepidermal/deep dermal). As no differences were found between the profiles of control and sham control wounds, these were pooled and compared with the profiles of antibody treated wounds or TGF-β treated wounds. As all comparisons were not orthogonal, the significance levels were adjusted by a factor of 3/4 using the procedure outlined by Girden (1992). Hence a nominal 0.0375 significance level was used in place of a 0.05 level.

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control wounds. By contrast, the angiogenic response of wounds treated with lower doses of either anti-TGFβ1 or anti-TGFβ1 plus anti-TGFβ2 and wounds treated with any of the doses of anti-TGFβ2 did not differ from the control wounds (Fig. 3).

Wounds treated with 10 ng/injection or 50 ng/injection of either TGF-β1 or TGF-β2 or TGF-β3 were more vascular than the control wounds. This effect was most marked with 50 ng/injection of TGF-β3 (Fig. 4).

**Orientation of collagen**

Fourteen day wounds were stained with Masson’s trichrome stain to highlight the orientation of collagen fibers. The orientation of collagen fibers in wounds treated with 1 µl or 5 µl/injection of anti-TGFβ1 plus anti-TGFβ2 was better than that of control wounds and more like that of the normal dermis. Wounds treated with 5 µl/injection of anti-TGFβ1 also showed some improvement in the orientation of collagen fibers. The orientation of collagen in wounds treated with lower doses of anti-TGFβ1 plus anti-TGFβ2 or with anti-TGFβ1 and in wounds treated with any of the doses of anti-TGFβ2 was similar to that in control wounds (Fig. 5).

The orientation of collagen fibers in wounds treated with 10 ng/injection or 50 ng/injection of TGF-β3 was better than the control wounds and more like that of the normal dermis. Wounds treated with all doses of TGF-β1 or TGF-β2 showed similar orientation of collagen to that in control wounds (Fig. 6).

The results of these dose response experiments showed that some effects on the various parameters examined were seen with 1 µl/injection but the best effects were obtained when wounds were injected with 5 µl/injection of the above neutralising antibodies. These experiments also showed that some effects were seen when wounds were treated with 10 ng/injection of the TGF-β isoforms; but these effects were more marked when 50 ng/injection of TGF-β isoforms were

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**Fig. 1.** Dose dependent response of isoform specific neutralising antibodies to TGF-β on the intensity of staining for fibronectin in healing wounds - a semiquantitative analysis. Sections of wounds harvested 7 days post-wounding were immunostained for fibronectin and the intensity of staining was scored on a scale of −4 to +4 (where 0=same as normal dermis; 1=1-25% of normal dermis; 2=26-50% of normal dermis; 3=51-75% of normal dermis; 4=>75% of normal dermis; prefix + = more than; and prefix − = less than.) Values are means of 6 observations (number of animals per group=2). Wounds treated with either 1 µl/injection or with 5 µl/injection of neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 stained less intensely for fibronectin than did the control wounds. Wounds treated with 5 µl/injection of either neutralising antibody to TGF-β1 or with neutralising antibody to TGF-β2 also stained less intensely than the control wounds. In the deep dermal region the intensity of staining for fibronectin in wounds treated with 5 µl of anti-TGF-β1+2 was similar to normal dermis i.e. score=0. Wounds treated with lower doses of the isoform specific neutralising antibodies to TGF-β did not differ from the control wounds.
used. For all subsequent experiments 20 ng/injection of TGF-β isoforms was used unless otherwise stated.

The next set of dose response experiments were established with wounds being treated with either 5 µg/injection or 50 µg/injection of commercially available neutralising antibody to TGF-β1 and neutralising antibody to TGF-β2. Wounds treated with 50 µg/injection of these neutralising antibodies gave a better response than those treated with 5 µg/injection. The effects of these commercial antibodies were similar to those seen with the antibodies supplied by Dr A. B. Roberts (Laboratory of Chemoprevention, NCI, Bethesda, USA). The dose dependent effects of 50 µg/injection and 5 µg/injection of the commercial antibodies corresponded with the effects of 5 µl/injection and 1 µl/injection, respectively, of the neutralising antibodies used in the previous set of experiments. Wounds treated with 50 µg/injection of neutralising antibody to TGF-β1 plus 50 µg/injection of neutralising antibody to TGF-β2 showed a marked reduction in immunostaining for fibronectin, a lower angiogenic response and on day 14 post-wounding, had a better orientation of collagen fibers in the neodermis than the control wounds. These effects were less marked in wounds treated with 5 µg/injection of these antibodies. Therefore for all subsequent experiments, the dose of each of the neutralising antibodies used was 50 µg/injection (Fig. 7).

Effects of modulating the TGF-β isoform levels in healing wounds

Inflammatory response

The macrophage and monocyte profile of 3 and 7 day healing wounds was studied as a paradigm of the inflammatory response. During the first 7 days post-wounding, wounds treated with anti-TGFβ1 plus anti-TGFβ2 had a significantly lower macrophage and monocyte profile than did the control wounds (P<0.01). Treatment with anti-TGFβ1 had some effect on lowering this profile in the subepidermal region of the wounds but not in the deep dermal region. By contrast, exogenous addition of either anti-TGFβ2 or anti-TGFβ1+2+3 did not alter the macrophage and monocyte profile of healing wounds markedly (Fig. 8).

The monocyte and macrophage profile of wounds treated with TGF-β3 was significantly lower than that of control wounds during the first 7 days post-wounding (P<0.01). By contrast, exogenous addition of TGF-β1 or TGF-β2 did not alter the monocyte and macrophage profile of healing wounds significantly (Fig. 9).

Angiogenesis

The vascularity of healing wounds assessed on day 7 post-wounding showed that wounds treated with anti-TGFβ1 plus anti-TGFβ2 were less vascular than the control wounds. By contrast, exogenous addition of anti-TGFβ1 or anti-TGFβ2 or anti-TGFβ1+2+3 to wounds had no effect on the vascularity when compared with the corresponding control wounds.

Wounds treated with TGF-β1, TGF-β2 or TGF-β3, showed an increase in angiogenic response compared to control wounds on day 7 post-wounding. However, this increase in vascularity was more marked in wounds treated with TGF-β3 than in TGF-β1 treated wounds whilst TGF-β2 treated wounds were only marginally more vascular than the control wounds.

Extracellular matrix deposition

Fibronectin

Fibronectin deposition was assessed on day 7 post-wounding. Exogenous addition of anti-TGFβ1 plus anti-TGFβ2 resulted in less fibronectin deposition compared to the control wounds. This decrease was seen in both the subepidermal and deep dermal regions of the wounds but was more marked in the deep dermal region. Wounds treated with anti-TGFβ1 also showed reduced intensity of staining for fibronectin although, this was not as marked as in wounds treated with anti-TGFβ1 plus anti-TGFβ2. Exogenous addition of anti-TGFβ2 did not alter the
Fig. 3. Dose dependent effects of isoform specific neutralising antibodies to TGF-β on the angiogenic response of healing wounds. Sections of wounds harvested on day 7 post-wounding and immunostained for von Willebrand factor to mark the blood vessels. Wounds treated with either 5 µl/injection of neutralising antibody to TGF-β1 (5) or with 5 µl/injection of neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 (7) have less blood vessels at the wound site compared to the control wound (4). Vascularity of wounds treated with lower doses of neutralising antibody to TGF-β1 (1) or neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 (3) or with any dose of neutralising antibody to TGF-β2 (2,6) is similar to that of control wounds. (C, control wound; A, neutralising antibody to TGF-β treated wounds; subscripts 1,2,1+2 indicate TGF-β isoform specificity; 0.1 µl, 1 µl, 5 µl indicate the dose of the neutralising antibody to TGF-β per injection).
Fig. 4. Dose dependent effects of TGF-β isoforms on the vascularity of healing wounds. Sections of wounds harvested 7 days post-wounding were immunostained for von Willebrand factor to mark the blood vessels. Wounds treated with 10 ng/injection or 50 ng/injection of TGF-β1 (1 and 5), TGF-β2 (2 and 6) or with TGF-β3 (3 and 7) are more vascular than the control wound (4). The increase in vascularity is most marked in wounds treated with 50 ng/injection of TGF-β3 (7). (C, control wound; T, TGF-β treated wounds; subscripts 1,2,3 indicate the isoforms; 10 ng and 50 ng indicate the dose of TGF-β/injection.)
fibronectin deposition in the subepidermal region but had a small effect in reducing the intensity of staining for fibronectin in the deep dermal region of the wounds. By contrast, the intensity of staining for fibronectin in wounds treated with neutralising antibody to TGF-β1+2+3 was similar to that of control wounds (Fig. 10; Table 3).

The intensity of staining for fibronectin in wounds treated with TGF-β3 was less than in control wounds on day 7 post-wounding. This decrease was more marked in the deep dermal region of the wounds. By contrast, treatment with TGF-β1 or with TGF-β2 increased the intensity of staining for fibronectin compared to that of control wounds. This effect was more marked with TGF-β1 than with TGF-β2 and more in the subepidermal than in the deep dermal region of the wounds (Fig. 11, Table 3).

Collagen I

Collagen I deposition was assessed on days 7 and 70 post-wounding. All wounds harvested on day 7 post-wounding stained less intensely for collagen I than did the surrounding normal skin. Wounds treated with anti-TGFβ1 plus anti-TGFβ2 or with anti-TGFβ1 alone, stained less intensely for collagen I than did the control wounds on day 7 post-wounding. This difference was more marked in the deep dermal region of the wounds. Wounds treated with anti-TGFβ2 stained slightly more intensely than the control wounds in the subepidermal regions of the wounds but the collagen I staining in the deep dermal region of the wounds was similar to that in the control wounds. By contrast, the intensity of staining for collagen I in wounds treated with neutralising antibody to TGF-β1+2+3 was similar to that of control wounds (Table 3).

Wounds treated with TGF-β3 stained less intensely for collagen I than control wounds on day 7 post-wounding. This difference was more marked in the deep dermal than in the subepidermal region of the wounds. By contrast, the intensity of staining for collagen I in wounds treated with TGF-β1 or with TGF-β2 was marginally more intense than in control wounds. This increase was more noticeable in the subepidermal than in the deep dermal region of the wounds (Table 3).
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The intensity of staining for collagen I in all wounds was similar to that of the surrounding dermis on day 70 post-wounding.

Collagen III

Collagen III deposition was assessed on days 7 and 70 post-wounding. Immunostaining for collagen III in the subepidermal region of control wounds was more intense than in the subepidermal region of the surrounding normal dermis whilst the deep dermal region of control wounds stained less intensely than the deep dermal region of normal dermis, on day 7 post-wounding. Wounds treated with anti-TGF-β1 plus anti-TGF-β2 or with anti-TGF-β1 alone, stained less intensely for collagen III than did the control wounds on day 7 post-wounding. This difference was more marked in the deep dermal region of the wounds. By contrast, the intensity of staining for collagen III in wounds treated with anti-TGF-β2 or with anti-TGF-β1+2+3 was similar to that of control wounds.

Wounds treated with TGF-β3 stained less intensely than the

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<td>SE</td>
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</table>

Values are means (12 observations; n=4) of scores for intensity of fibronectin collagen I staining in wounds harvested 7 days post-wounding. Scale: 0, same as normal dermis; 1, 1-25% of normal dermis; 2, 26-50% of normal dermis; 3, 51-75% of normal dermis; 4, >75% of normal dermis; no prefix, more than and prefix, -, less than. SE, subepidermal region; DD, deep dermal region.

Fig. 7. Dose dependent effects of neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 on fibronectin deposition in healing wounds. Sections of wounds harvested 7 days post-wounding and immunostained for fibronectin. The isoform specific neutralising antibodies to TGF-β used in this experiment were from British Biotechnology Ltd, Oxford. The intensity of staining for fibronectin in wounds treated with neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 (2 and 4) was less than that in the control wounds (1 and 3). This reduction was more marked in wounds treated with 50 µg/injection of the neutralising antibodies compared to wounds treated with 5 µg/injection of the neutralising antibodies. (C, control wounds; A, neutralising antibody treated wounds; subscript 1+2 indicates TGF-β isoform specificities; 5 µg and 50 µg indicate dose of the antibody/injection).
control wounds and this decrease in collagen III was more marked in the subepidermal region of the wounds. By contrast, wounds treated with either TGF-β1 or with TGF-β2 stained more intensely than control wounds (Fig. 12). Immunostaining for collagen III in all wounds was similar to that in the normal skin on day 70 post-wounding.

Architecture of the neodermis
The architecture of the neodermis was assessed on day 70 post-wounding. The architecture of the neodermis in wounds treated with anti-TGFβ1 plus anti-TGFβ2 was markedly better than that seen in control wounds i.e. it looked more like normal dermis in terms of orientation, spacing and size of collagen bundles. Wounds treated with anti-TGFβ1 also showed some improvement, but this was not as marked as in wounds treated with anti-TGFβ1 plus anti-TGFβ2. By contrast, the architecture of the neodermis in wounds treated with anti-TGFβ2 or with anti-TGFβ1+2+3 was similar to that of control wounds (Fig. 13).

The architecture of the neodermis in wounds treated with TGF-β3 was markedly better than that seen in control wounds and, barring the absence of hair follicles, resembled closely that of normal dermis. The improvement was more marked in wounds treated with 50 ng/injection of TGF-β3. By contrast, the architecture of the neodermis in wounds treated with TGF-β-

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Fig. 8. Effect of isoform specific neutralising antibodies to TGF-β on the monocyte and macrophage profile of healing wounds. Sections of wounds harvested on days 3 and 7 post-wounding were immunostained with a monoclonal antibody ED1 to mark the monocytes and macrophages. An image analyser was used to count the number of positively stained macrophages and monocytes within a defined area (0.162 mm²) as described in Materials and Methods. Values represent means of 4 observations and the error bars represent one standard deviation. Wounds treated with neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 have a significantly lower monocyte and macrophage profile than the control wounds during the first week post-wounding (P<0.01). Wounds treated with neutralising antibody to TGF-β1 have a lower monocyte and macrophage profile than the control wounds in the subepidermal region. By contrast, neutralising antibody to TGF-β2 or neutralising antibody to TGF-β1+2+3 did not alter the monocyte and macrophage profiles of healing wounds.
β2 or with TGF-β1 was similar to that of control wounds i.e. scarred (Fig. 14).

For all parameters studied, the sham control wounds were similar to the control wounds.

**Macroscopic appearance of healed wounds**

There were marked differences in the macroscopic appearance of healed wounds 70 days after wounding. The sites of the control, sham control, anti-TGFβ2, anti-TGFβ1+2+3, TGF-β1 and TGF-β2 treated wounds were replaced by white, shiny, firm and raised scars. The sites of the wounds treated with anti-TGFβ1 had flat, firm, scars. By contrast, it was very difficult to locate the sites of TGF-β3 treated or anti-TGFβ1 plus anti-TGFβ2 treated wounds after clipping the fur, due to a marked reduction in scarring (Fig. 15).

**DISCUSSION**

This study has shown that exogenous addition of neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 to incisional, cutaneous wounds in adult rodents reduces scarring. Neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 reduced the monocyte and macrophage profile, neo-vascularisation, fibronectin, collagen III and collagen I deposition in the early stages of wound healing and improved the architecture of the neodermis, thereby reducing scarring. These effects were similar to those reported earlier where a polyclonal neutralising antibody to TGF-β (not isoform specific) reduced scarring in adult rodent wounds (Shah et al., 1992, 1994). Exogenous addition of neutralising antibody to TGF-β1 resulted in only a marginal reduction in scarring. By contrast,
Fig. 10. Effects of isoform specific neutralising antibodies to TGF-β on fibronectin deposition in healing wounds. Sections of wounds harvested on day 7 post-wounding and immunostained for fibronectin. Wounds treated with neutralising antibody to TGF-β1 (1) or with neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 (3) showed less intensity of staining for fibronectin than did the control wound (5). The reduction in intensity of staining for fibronectin was more marked in wounds treated with neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 (3). The intensity of staining in wounds treated with neutralising antibody to TGF-β2 (2) was less than that in the control wounds only in the deep dermal region. By contrast, the intensity of staining for fibronectin in wounds treated with the neutralising antibody to TGF-β1,2,3 (4) did not differ from that in the control wound (C, control wound; A, neutralising antibody treated wound; subscripts 1,2,3 indicate TGF-β isoform specificity).
exogenous addition of neutralising antibody to TGF-β₂ or neutralising antibody to TGF-β₁+₂+₃ to wounds did not alter scarring. None of the treatments delayed wound healing.

These experiments suggest that TGF-β₁ and TGF-β₂ both contribute to the repair process of adult wound healing and subsequent scar formation. Of the two isoforms, TGF-β₁ appears to play a greater role in extracellular matrix deposition and scar formation. Treatment with neutralising antibody to TGF-β₁ resulted in less fibronectin, collagen III and collagen I deposition compared to control wounds as assessed by immunohistochemical studies. Although immunohistochemistry is not a quantitative assay, previous studies (Shah et al., 1994) have demonstrated a correlation between the collagen content of wounds analysed biochemically and immunohistochemically. The architecture of the neodermis as well as the macroscopic appearance of the scar were slightly better than those of the control wounds. Treatment with neutralising antibody to TGF-β₂ only marginally reduced extracellular matrix deposition but did not alter the final scar. These experiments suggest that it is essential to reduce both TGF-β₁ and TGF-β₂ levels in healing wounds in order to achieve scarfree healing. This may imply some synergistic interaction of TGF-β₁ and TGF-β₂ in the process of scar formation.

Immunolocalisation and gene expression studies of the three TGF-β isoforms during embryogenesis have shown distinct temporal and spatial distribution of these isoforms (Pelton et al., 1990, 1991; Gatherer et al., 1990; Millan et al., 1991; Schmid et al., 1991). It has been proposed that TGF-β₁ might be involved in the early inductive events of epithelial-mesenchymal interactions and formation of the extracellular matrix components important for cell migration and cell-cell interaction (Schmid et al., 1991). TGF-β₂ may be involved in the differentiation and homeostasis of differentiated cells whilst TGF-β₃ could be involved in morphogenesis as well as in differentiation (Pelton et al., 1990, 1991; Gatherer et al., 1990; Millan et al., 1991; Schmid et al., 1991).

Wound healing like embryogenesis is a complex process involving cell migration, proliferation, differentiation and extracellular matrix formation. It is tempting to speculate that the three isoforms of TGF-β may be playing different roles in wound healing similar to their effects in embryogenesis. TGF-β₁ released by degranulating platelets may be involved in the early induction of the healing process. It attracts monocytes, macrophages, other inflammatory cells and fibroblasts to the wound site and induces them to produce potent mitogens such as PDGF and basic FGF and also autoinduces the production of TGF-β₁ thus perpetuating its presence at the wound site. Under the influence of PDGF and FGF, fibroblasts, endothelial cells and epithelial cells proliferate. TGF-β₁ induces deposition of the extracellular matrix components necessary for cell

**Fig. 11.** Isoform specific effects of TGF-β on fibronectin deposition in healing wounds. Sections of wounds harvested on day 7 post-wounding and immunostained for fibronectin. Wounds treated with TGF-β₁ (2) or with TGF-β₂ (4) show a marked increase in the intensity of staining for fibronectin compared to that in the control wound (1). By contrast, the intensity of staining for fibronectin in wounds treated with TGF-β₃ (3) is markedly less than that in the control wound (C, control wound; T, TGF-β treated wound; subscripts 1,2,3 indicate TGF-β isoforms).
migration and cell-cell interaction as well as that of fibrillar collagens required for tissue integrity. However, the overdrive response of TGF-β1 may be downregulating the expression of TGF-β2 and TGF-β3 by fibroblasts (Bascom et al., 1989) or altering the TGF-β receptor profile, ultimately leading to scar formation.

Exogenous addition of neutralising antibody to TGF-β1 alone, did not reduce this overdrive response adequately whilst addition of neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 prevented this overdrive and improved scarring. TGF-β2 is known to upregulate the expression and secretion of TGF-β1 by monocytes, macrophages, fibroblasts and epithelial cells (Bascom et al., 1989a,b; McCartney-Francis et al., 1990; O’Reilly et al., 1992). TGF-β2 expression by monocytes is not altered by TGF-β1 therefore the presence of endogenous TGF-β2 may be responsible for some of the overdrive response of TGF-β1 even in the presence of neutralising antibody to TGF-β1. This may also explain why addition of neutralising antibody to TGF-β2 alone did not have a significant effect.

Previous studies on wound healing utilised exogenous application of either the TGF-β1 (Sporn et al., 1983; Mustoe et al., 1987) or TGF-β2 (Ksander et al., 1990) isoform alone and reported an increased rate of healing as assayed by tensile strength measurements and the histological maturity of wounds. These findings are in keeping with the immunocytochemical observations reported here. However, our findings of the antiscarring effects of TGF-β3 are novel.

It may be that the beneficial effects of neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 are due to the relative increase in the amount of TGF-β3 present in the healing wounds and not just due to a reduction in the levels of TGF-β1 and TGF-β2. This could explain why no reduction in scarring was seen when wounds were treated with neutralising antibody to TGF-β1+2+3. This antibody also neutralises endogenous TGF-β3. The effect of this antibody was similar to the effect reported with the highest dose of the polyclonal neutralising antibody used in the previous experiments (Shah et al., 1994). The paradoxical effect seen with the high dose of this antibody may be due to neutralisation of more of the endogenous TGF-β3 with the higher dose of the antibody, and/or to the antibody acting like a binding protein thus elevating and prolonging the presence of TGF-β at the wound site.

Exogenous addition of TGF-β3 to cutaneous, incisional wounds resulted in a marked improvement in the architecture of the neodermis and a reduction in scarring. By contrast, treatment with TGF-β1 or with TGF-β2 did not alter scar formation. 

Fig. 12. Isoform specific effects of TGF-β on collagen III deposition in healing wounds. Sections of wounds harvested on day 7 post-wounding and immunostained for collagen III. Wounds treated with TGF-β1 (2) or with TGF-β2 (4) show an increase in the intensity of staining for collagen III compared to that in the control wound (1). By contrast, the intensity of staining for collagen III in wounds treated with TGF-β3 (3) is markedly less than that in the control wound (C, control wound; T, TGF-β treated wound; subscripts 1,2,3 indicate TGF-β isoforms).
Fig. 13. Effect of isoform specific neutralising antibodies to TGF-β on the architecture of the neodermis. Sections of wounds harvested 70 days post-wounding and stained with Picrosirius Red were photographed under identical settings using a polarising microscope. Note the basket-weave pattern of collagen in the dermis of normal, unwounded skin (2). By contrast, the collagen fibers in the neodermis of the control wound (1), neutralising antibody to TGF-β2 treated wound (5) and neutralising antibody to TGF-β1,2,3 treated wounds (6) are compactly arranged in an abnormal pattern with distinct scarring. The collagen fibers in the neodermis of wounds treated with neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 (4) are arranged in a reticular pattern closely resembling that of the normal dermis with a marked reduction in scarring. Wounds treated with neutralising antibody to TGF-β1 (3) show some reduction in scarring though the effect is not as marked as that seen in wounds treated with neutralising antibody to TGF-β1 plus neutralising antibody to TGF-β2 (C, control wound; N, normal skin; A, neutralising antibody treated wound; subscripts 1,2,3 indicate TGF-β isoform specificity; arrowheads indicate junction between normal dermis and the neodermis).
formation. Treatment of wounds with TGF-β3 resulted in a lower monocyte and macrophage profile, increased neovascularisation, lower fibronectin, collagen III and collagen I deposition in the earlier stages of healing and a marked improvement in the architecture of the neodermis, resembling the normal dermis and resulting in reduced scarring.

Most in vitro studies have shown similar biological activities of the three isoforms of TGF-β but with different potencies (Graycar et al., 1989; Roberts et al., 1990). The only similar biological activity of the three TGF-β isoforms seen in this in vivo study was the increase in angiogenesis. Exogenous addition of TGF-β3 appeared to have the most marked effect although in vitro studies have shown TGF-β3 to have an identical effect to TGF-β1 (Merwin et al., 1991). In keeping with the in vitro studies, TGF-β2 treated wounds showed only a marginal increase in neovascularisation (Merwin et al., 1991).

Exogenous addition of TGF-β3 significantly reduced the magnitude of monocyte and macrophage infiltration into healing wounds. However, in vitro assays for chemotaxis of

**Fig. 14.** Isoform specific effects of TGF-β on the architecture of the neodermis. Sections of wounds harvested 70 days post-wounding stained with Picrosirius Red and photographed under identical settings using a polarising microscope. The collagen fibers in the neodermis of control (1) and sham control (6) wounds, TGF-β1 treated wound (4) and TGF-β2 treated wound (5) are compactly placed in an abnormal pattern with distinct scars. By contrast, the architecture of the neodermis of wounds treated with TGF-β3 (3) closely resembles that of the normal dermis (2) with the collagen fibers loosely arranged in a basket weave pattern (C, control wound; S, sham control wounds; N, normal skin; T, TGF-β treated wound; subscripts 1,2,3 indicate the isoforms; arrowheads indicate junctions between normal dermis and neodermis).
human peripheral monocytes have shown that TGF-β₁ and TGF-β₃ are equipotent (Roberts et al., 1990). It may be that in certain situations such as wound healing where other cytokines are also present, pharmacological doses of TGF-β₃ may act as a negative regulator of inflammation. Alternatively in vitro assays may alter the isoforms of TGF-β receptors expressed and so alter the cells response to individual TGF-β isoforms. Equivalent doses of TGF-β₁ or TGF-β₂ did not alter the monocyte and macrophage profile of healing wounds suggesting that in the absence of infection, the normal wound healing process in the rodent has a redundant inflammatory response.

In the early stages of wound healing, TGF-β₃ treated wounds had less fibronectin, collagen III and collagen I whilst TGF-β₁ and TGF-β₂ treated wounds had more extracellular matrix compared to the control wounds. By reducing the monocyte and macrophage profile, TGF-β₃ may be reducing the TGF-β₁ and TGF-β₂ levels (Bascom et al., 1989a) resulting in reduction of extracellular matrix deposition. A more gradual and organised deposition of extracellular matrix probably influences the final architecture of the neodermis and hence also reduces scarring. TGF-β₃ may also have different effects on integrin expression thereby influencing the cellular infiltration and extracellular matrix production. To this end, in vitro studies have shown that at lower doses, TGF-β₃, unlike TGF-β₁ and TGF-β₂, does not antagonise migration of fibroblasts through a collagen gel under the influence of migration stimulating factor-MSF (Ellis et al., personal communication).

The recent cloning of TGF-β receptors and studies using cells overexpressing truncated type II receptors as dominant negative mutants have shown that type I receptors are responsible for the effects of TGF-β on the induction of extracellular matrix such as fibronectin and plasminogen activator inhibitor type I. Type II receptors, possibly in conjunction with type I receptors, mediate the antiproliferative effects of TGF-β (Chen et al., 1993). It will be interesting to study whether TGF-β₃ differentially regulates the expression of these TGF-β receptor types or different type I/type II complexes in the wound healing environment and thereby produces its antiscarring effects. In this respect, the isoform specific effects of TGF-β may be similar to those seen with another cytokine implicated in wound healing i.e. platelet derived growth factor (PDGF). PDGF occurs as a dimeric molecule consisting of two polypeptide chains A and B in all possible combinations which dimerise with α and β receptor subunits to form different dimeric receptor complexes (Heldin and Westermark, 1989). The responsiveness of different cell types to PDGF isoforms is thus dependent on the numbers and types of the receptors on the cell membrane. Whilst both receptors transduce mitogenic responses, their abilities to stimulate chemotaxis of monocytes, fibroblasts and granulocytes differ (Siegbahn et al., 1990). Both PDGF-AA and PDGF-BB significantly increased the number of granulation tissue derived fibroblasts in vitro; but, in vivo, only PDGF-BB significantly increased granulation tissue formation and collagen content of cellulose sponges implanted subcutaneously in rats while PDGF-AA had a non-significant effect (Lepisto et al., 1992).

The beneficial effects of endogenous TGF-β₃ appear to be masked by the overdrive response of TGF-β₁. It may be that for survival of the adult organism, rapid closure with prevention of infection and dehiscence of the wound take precedence over regeneration and restoration of the dermal architecture resulting in scar formation.

This study has shown that both TGF-β₁ and TGF-β₂ are involved in the process of scar formation after cutaneous wounding in the adult rodent. By contrast, TGF-β₃ improves the dermal architecture and reduces scar formation. The exact mechanism by which TGF-β₃ reduces scarring remains to be elucidated. However, the use of TGF-β₃ as an anti-scarring agent seems promising.

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