Hypertrophic (HTS) scars are a common complication of wound healing, characterized by excessive collagen deposition and prolonged cellularity. The precise mechanisms underlying the increased collagen synthesis and low cellularity of hypertrophic scars are not fully understood. Previous studies have suggested that fibroblasts from hypertrophic scars exhibit altered responsiveness to mitogens compared to normal skin fibroblasts. These differences in cytokine responsiveness may contribute to the phenotypic changes observed in hypertrophic scars.

Materials and Methods

**Cells** Fibroblasts were isolated from hypertrophic and normal skin samples obtained from burn patients. The fibroblasts were cultured in standard conditions and their response to various mitogenic factors was assessed.

**Methods** The fibroblasts were exposed to different concentrations of platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and tumor necrosis factor α (TNFα) to evaluate their mitogenic potential. The collagen synthesis rate was determined using a hydroxyproline assay, and cell viability was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Results

Fibroblasts from hypertrophic scars showed a significantly lower collagen synthesis rate compared to normal skin fibroblasts, despite a similar mitogenic response to PDGF and EGF. This suggests that hypertrophic scars exhibit a specific alteration in the collagen synthesis pathway.

Discussion

The findings support the hypothesis that altered cytokine responsiveness contributes to the phenotypic differences observed in hypertrophic scars. These results have implications for the development of targeted therapies aimed at modulating fibrotic processes in wound healing.

Keywords: hypertrophic scars, fibroblasts, cytokines, collagen synthesis.
experiments or passaged after brief trypsinization using a one-to-three split ratio. All studies reported here were performed only on cells at passages ≤ 3.

Mitogenesis Assay Mitogenic response to serum and cytokines was determined essentially as previously described [16]. Briefly, 10^4 fibroblasts in MCDB 110 containing 5% FBS were plated into each well of a 96-well microtiter plate. After overnight incubation, the media were removed, the cells washed, and the media replaced with MCDB 110 containing 0.4% FBS. After 24 h, the cells were exposed to the indicated doses of either FBS, platelet-derived growth factor (PDGF, from human platelets, specific activity 500 units/mg; Upstate Biotechnology, Lake Placid, NY), epidermal growth factor (EGF, from mouse submaxillary gland; Sigma), or tumor necrosis factor α (TNFα, specific activity > 2 × 10^9 units/ml; R&D Systems, Minneapolis, MN) for another 24 h. During the last 6 h of incubation, the cells in each well were pulsed with 1.0 μCi of [H]-thymidine (specific activity 30 Ci/mmol; ICN Biomedicals, Irvine, CA). The cells were then trypsinized and transferred onto glass-fiber filters using an automated harvester (Brandel, Gaithersburg, MD). After washing, the filters were counted for radioactivity and data were expressed as dpm incorporated per well.

Collagen Synthesis Assay Protein synthesis was measured as previously described [17]. Briefly, 10^5 cells were plated into each well of a 12-well tissue culture plate (22 mm in diameter) and allowed to adhere overnight. The media were then removed, and replaced with serum-free media supplemented with 10% FBS (Sigma). After 24 h incubation, the cells were treated with the indicated concentrations of transforming growth factor β1 (TGFβ1, porcine platelet-derived; R&D Systems, Minneapolis, MN) for 48 h in media supplemented with 50 μg/ml sodium ascorbate and 2 mg/ml bovine serum albumin (BSA). During the last 6 h of incubation, the cells were pulsed with 5 μCi/ml [H]-proline (specific activity 93 Ci/mmol, Amersham Radiochemicals, Arlington Heights, IL) in the presence of β-aminoisobutyric acid. The media and one wash were then collected and analyzed for radioactivity in collagenous and noncollagenous proteins, as defined by susceptibility to degradation by purified bacterial collagenease [18]. Under these conditions > 80% of the newly synthesized collagenous proteins are secreted into the media (data not shown). The cell layer in each well was harvested by trypsinization and cell number determined using a Coulter counter. Protein synthetic rates were expressed as dpm incorporated by 10^5 cells during the 6-h pulse. The fraction of protein synthesis devoted to collagen or percent collagenous protein synthesis was calculated as previously described after correcting for the lower average content of proline in non-collagenous proteins [19].

Data Analysis Statistical comparisons of mitogenic and protein synthetic responses were performed on mean values for each cytokine dosage using analysis of variance (ANOVA). When significant differences were found, the method of Tukey (SAS Institute, Inc., Cary, NC) was used for individual comparisons. Where necessary to meet the normal distribution requirement for the use of ANOVA, the data were log-transformed prior to application of ANOVA and Tukey's Studentized range test.

RESULTS

Mitogenic Responsiveness to Serum and Cytokines There were no significant differences in basal (0.4% FBS) rates of thymidine incorporation between HTS and NS fibroblasts from the same subject. As there is some variation in absolute rates of thymidine uptake from day to day, and significant heterogeneity in the patient population, all values were normalized to the dpm taken up by their own respective control cells exposed only to 0.4% FBS.

When expressed in this manner, the mitogenic response of HTS fibroblasts was not significantly different from NS cells at doses of FBS < 2.5% (Fig 1). At doses ≥ 2.5% FBS, the NS cells were significantly more responsive compared to HTS cells, except for the highest dose (20%) tested, when the differences disappear (Fig 1). Peak stimulation of thymidine uptake in both HTS and NS cells occurred at 2.5–5% FBS, a standard result for primary human dermal fibroblast cultures in this laboratory. Decreasing stimulation is seen with higher levels of serum supplementation. At 20% FBS, HTS fibroblasts showed no increased uptake above baseline. The maximal difference between HTS and NS fibroblasts occurred at 2.5% FBS, when the HTS response was approximately 64% of normal.

EGF is present in platelets [20] and hence represents one of the mitogens present in serum. To evaluate whether it could be one of the mitogens responsible for the distinction observed between HTS and NS in their response to FBS, the effects of pure authentic EGF were examined. The responses of HTS and NS fibroblasts to EGF are shown in Fig 2. There was a significant increase in thymidine uptake by both HTS and NS cells when stimulated with EGF, including that at the lowest dose tested, namely 0.5 ng/ml. The shapes of the dose-response curves for both HTS and NS cells to this mitogen were similar, but the magnitudes of the responses were significantly greater in NS cells at all doses of EGF indicated in Fig 2. As a result of EGF stimulation, incorporation averaged 700% of control for NS and 420% of control for HTS. The maximal EGF-induced mitogenic stimulation is approximately twice as great for NS as for HTS fibroblasts, which was comparable to that seen at the peak of FBS (2.5% dose) stimulation. EGF doses greater than 8 ng/ml failed to cause any further increase in the mitogenic response of both HTS and NS cells (data not shown).

PDGF is a cytokine with well-known mitogenic properties, and has been implicated in the pathogenesis of fibrosis [21,22]. It is also present in platelets [20] and thus represents another important mitogenic component of serum. To determine whether PDGF also plays a role in the ability of serum to discriminate between HTS and NS cells, the effects of authentic PDGF were also studied. The mitogenic responses to varying doses of PDGF show an increase in thymidine uptake with increasing concentrations of PDGF in both NS and HTS. In contrast to the EGF dose-response curves, however, the responsiveness of HTS was not significantly different from that of NS cells at all doses of PDGF studied (data not shown). This would suggest that the discriminating properties of serum were not due to its PDGF component.

TNFα is primarily produced by macrophages and monocytes, which are known to be present at sites of injury and inflammation [23,24]. It is known to be important in fibrotic responses in other organ systems, including the skin [25]. It is known to have growth-promoting properties in vitro and thus represents another type or class of growth factor [26]. Therefore the effects of this cytokine on fibroblast proliferation were evaluated to see whether this cytokine could also distinguish the HTS from NS cells. The results of TNFα stimulation show that the dose-response curves were similar in shape to those for EGF, but the magnitudes of the responses by HTS cells to TNFα were not significantly different from those for NS cells at all doses (0–10 ng/ml) studied (data not shown). The HTS cell responses at the indicated doses of TNFα ranged from 70–90% of those for NS cells. This suggests that at the site of injury, increased production of TNFα is unlikely to affect the selection of fibroblast subpopulation(s) with the HTS phenotype.

Figure 1. Mitogenic response to FBS. HTS and NS fibroblasts were compared for [H]-thymidine uptake after stimulation with the indicated doses of FBS as described in Materials and Methods. The mitogenic response of NS fibroblasts was significantly greater than that of HTS cells only at FBS concentrations of 2.5, 5, and 10% (p < 0.05). Data represent the mean dpm (± SEM, n = 11) taken up per well after expression as a percentage of the mean dpm taken up by the respective control cells exposed only to 0.4% FBS. Baseline incorporation by these control cells ranged from 1000 to 2500 dpm per well.
Collagen Synthetic Phenotype

Hypertrophic scars are characterized by the increased content of the extracellular matrix, including collagen [27]. Although the data are conflicting with regard to the demonstration of increased collagen synthesis by scar tissue, the end result of increased collagen deposition is clear [28]. This increased collagen could be the result of increased synthesis, and is perhaps connected with decreased breakdown. To examine whether such an increased rate of collagen synthesis could be a phenotypic characteristic of the HTS cells, the basal and TGFβ1-stimulated rates of collagen synthesis in NS and HTS cells were measured. The basal or non-stimulated rate of collagen synthesis was significantly greater in HTS fibroblasts compared to their matched NS cells. In addition, when these cells were stimulated with 0.5 and 1.0 ng/ml of TGFβ1, the collagen synthetic response of HTS fibroblasts remained significantly greater (Fig 3). When a higher dose of TGFβ1 (2 ng/ml) was used, the NS cells almost doubled their collagen production, whereas the responses in HTS cells appear to show no further significant increases or may decline, compared to those at 0.5 and 1.0 ng/ml TGFβ1. Consequently, the difference in collagen synthesis between HTS and NS cells became insignificant at 2 ng/ml TGFβ1. In contrast to collagen synthesis, the synthesis of secreted noncollagenous proteins was not significantly different between HTS and NS cells, both basally and in the presence of the same doses of TGFβ1 (data not shown). As a consequence, the percentage of protein synthesized as collagen was significantly greater in HTS (25.6 ± 1.1) versus NS cells (21.4 ± 1.3) (p < 0.02).

DISCUSSION

Because basic understanding of the pathogenesis of HTS is lacking, the present study attempts to document potential abnormalities in the fibroblasts contained within these lesions. The data show that fibroblasts isolated from resected HTS lesions have distinguishing phenotypic features from those isolated from normal unaffected skin of the same individual. Although both HTS and NS cells responded to known growth factors, the HTS cells were significantly less responsive to selected doses of serum and EGF. However, the mitogenic response to another growth factor normally present in serum, namely PDGF, was not significantly different between the two cell populations. This suggests that the differential response to serum may be primarily due to the EGF present in serum. TNFα is another cytokine known to be present in injured and inflamed tissue, and has been reported to also stimulate cell proliferation [26,29]. As with PDGF, it was also unsuccessful in discriminating HTS from NS cells. The basis for this selective effect of EGF is unclear, but may be related to decreased expression of EGF receptors in the HTS cells and/or diminished signal transduction in the HTS cells. This diminished responsiveness to a common growth factor may be important in accounting for the decreased cellularity of the mature HTS lesion. Further studies are necessary to uncover the mechanistic basis for this observation.

In contrast to mitogenic responsiveness, the HTS cells were upregulated with respect to collagen production when compared to NS cells. This elevated level of collagen production was observed both in the absence of added exogenous stimuli and in the presence of ≤ 1 ng/ml of TGFβ1, with the difference dissipating at higher doses due to the increased production by NS cells. The basis for this elevated rate of collagen production is also unknown, but may be related to autocrine stimulation by a heightened rate of endogenous TGFβ1 gene expression by the HTS cells themselves. This relatively stable phenotypic alteration is consistent with the observation of increased matrix deposition in HTS lesions, thus suggesting a role for this property in the genesis of such lesions.

Because the changes in fibroblast phenotype occurred in cells derived from lesions 1–12 years after burn injury, it is unlikely that these findings reflect the direct activity of mediators released into the healing burn wound. Moreover, many of these scars had a stable appearance during the previous months, suggesting that the acute phase of wound healing was complete and that direct effects of mediators released during this period would also have waned. Although it is possible that a long-standing mediator release into the local wound environment would result in the described changes in cell function, this possibility is unlikely because the phenotype was maintained in culture. The cells used for these studies were at passages 2–3, or after 3–5 population doublings away from primary culture with a 1:3 split ratio at each passage. After this number of cell divisions, the acute responses to a local mediator would be expected to decrease, and basal cell responses to be expressed.

The altered phenotype of HTS cells may be a result of selection and/or prolonged exposure to mediators present in injured skin. Local wound environment may encourage or diminish the growth of specific clones of fibroblasts, thus selecting for or against one or more subpopulations, thus yielding cells with the final phenotype observed in this study. Alternatively, specific events, such as cytokine release during burn wound healing, might permanently alter cellular responses, resulting in cell populations with a different phenotype. The latter mechanism, however, has not been documented, and furthermore there is no evidence that these cells are trans-
The heterogeneity of human dermal fibroblasts has been found to be related, in part, to anatomic location within the dermis. Cells from the superficial (papillary) dermis exhibit greater proliferative capacity than those derived from reticular dermis [30]. Whereas collagen synthetic response is not different between these tissue regions, type I procollagen was increased in medium from cultured reticular fibroblasts. This finding may be relevant to the clinical finding that HTS is more common after deeper burn injury and may lead to the specific findings of this study. After deep burn injury the healing wound is populated by cells with an intrinsic potential to synthesize more collagen [38]. If the healing wound is populated by such cells the normal matrix homeostasis would be disturbed, and more matrix protein would result.

Fibroblasts cultured from patients with scleroderma, a systemic disease associated with skin fibrosis, display an increased synthesis of matrix proteins and altered cytokine responses [7–9]. This phenotype could be a result of prolonged exposure to monocyte-derived factors, which have been shown to persist even after removal of the cells from the influence of these factors [15]. The implication is that changes in the dermal fibroblast phenotype may be cytokine driven by tissue monocytes. Studies of pulmonary fibrosis have documented increased lung fibroblast collagen synthesis by cells isolated from fibrinous lungs. Experimentally, this has been related to stimulated endothelial cell expression of TGF-β [39,40]. Dermal cells, including keratinocytes, are known to produce TGF-β [41,42]. The phenotype of HTS fibroblasts is similar to that seen after TGF-β exposure, suggesting a role for this cytokine in the HTS alteration of the dermal fibroblast phenotype. This conclusion is supported by the loss of differences in collagen synthesis at the higher TGF-β dose. Studies are currently in progress to determine the role of TGF-β in inducing the documented changes in cell phenotype. A variety of other stimuli may also cause increased local deposition of collagen. For instance, early scar formation may subsequently decrease the relative vascularity of the burn wound. Measurements of the tissue PO2 in fibrinous skin show significant decreases compared to normal skin [43]. Because hypoxia stimulates TGF-β synthesis [44,45], this may result in increased collagen synthesis and persistence of fibrosis. Cell clones able to tolerate this environment would persist, resulting in the selected phenotype reported in this study.

Although these data document an in vitro cellular difference between HTS and NS cells, the functional or pathophysiologic significance of this finding is unclear. Studies of phenotypic differences in cells isolated from serial biopsies along with clinical and functional correlation will be necessary to accurately determine their significance.

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