Introduction

The process of wound healing is initiated in the skin when there is a disturbance in the form of surgical injury or trauma of any sort, such as a burn. Wound healing is a process involving a delicate equilibrium between synthesis and degradation of extracellular matrix (ECM) components. In certain individuals, owing to certain racial and genetic factors, matrix deposition during wound healing is exuberant, resulting in scars that are unsightly and problematic for treatment. These scars are usually either hypertrophic or keloid, or they belong to an indeterminate group. Keloids and hypertrophic scars are fibroproliferative disorders resulting from abnormal wound healing. Clinically, keloids are defined as scars that extend beyond the margins of the original wound, while hypertrophic scars remain confined. Keloids can occur spontaneously in individuals with a predisposition to keloid formation. They recur after surgical excision or with skin grafting and do not regress with time. Hypertrophic scars, on the other hand, can be corrected by surgical procedures and they regress or flatten over a period of time. Apart from these well-defined forms of scars, there is an indeterminate group of scars with a massive, raised, keloid-like appearance that do not recur after surgical excision. Thus, clinical evaluation based merely on appearance is not very helpful for the differentiation of aberrant scars.

Although the process of wound healing has been well studied, the underlying mechanism leading to the formation of abnormal scars remains obscure. However, it is known that any injury to the reticular layer of the dermis leads to the formation of such scars, and it is in this region that fibroblasts reside. Hence, dermal fibroblasts are considered to be key players in scar formation. Apart from fibroblasts, ECM components also participate in scar formation. Scar tissue is characterized by overabundant ECM deposition in the dermal region of the skin and by an abnormal response of fibroblasts to growth modulators. However, in spite of these known aspects of abnormal wound healing, it is difficult for clinicians to differentiate such scars. A study based on the ultrastructural examination of ECM in the dermal region was therefore undertaken to examine the basic differences between the dermis of hypertrophic scars, keloids, and normal skin. The ECM of scar biopsies and normal skin biopsies was analysed using biochemical, histochemical, and ultrastructural methods. The results of this study could contribute to the armamentarium of physicians in the prevention of the formation of such scars and help to develop a treatment protocol.

Materials and methods

Clinical specimens

Tissues were obtained from the cosmetic surgery de-
partment of K.K. Childs Trust Hospital and Kilpauk Medical College and Hospital. Informed consent was obtained from each patient. All the patients providing tissue for this study were of Indian origin. The lesions were diagnosed as keloids or hypertrophic scars on the basis of clinical appearance and the history of the lesions.

**Water content**

The tissue specimens were immediately weighed for wet weight determination and lyophilized to obtain a constant dry weight. The difference between wet and dry weights was used as an estimate of water content.

**Estimation of collagen**

Estimation of hydroxyproline was used as a measure of collagen content in the tissues. Samples were hydrolysed in 6N HCl in sealed tubes at 110°C for 22 h. The hydrolysed samples were evaporated to dryness in a boiling water bath to remove acid, and the residue was dissolved in distilled water and made up to a known volume. Hydroxyproline was determined by the method of Woessner.⁷

**Estimation of proteoglycans**

Proteoglycans are made up protein cores that are heavily glycosylated with glycosamino glycans (GAGs). GAG estimation was therefore used as a measure of proteoglycan content in the tissues. GAGs were estimated by their hexosamine content. Samples were hydrolysed in 2N HCl in sealed tubes at 110°C for 6 h. The hydrolysed samples were evaporated to dryness in a boiling water bath to remove acid, and the residue was dissolved in distilled water and made up to a known volume. The total hexosamine content was estimated by the method of Elson and Morgan.⁷

**Preparation of tissues for electron microscopy**

The tissues and cell pellets (fibroblasts isolated from biopsies) were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, at room temperature. Post-fixation in 1% osmium tetroxide in phosphate buffer was carried out for a further period of 3 h at room temperature. Tissues fixed in osmium tetroxide were then washed, dehydrated through ascending grades of acetone, and embedded in araldite in flat embedding models, appropriate care being taken in orienting the tissues. Ultrathin sections were cut on the LKB ultratome V fitted with a glass knife and collected on copper grids. These grids were stained with 2% uranyl acetate and post-stained with 2% lead citrate solution. Stained grids were examined using a JEOL 100CX electron microscope.⁸

**Isolation of collagen**

Skin and scar biopsies from human subjects were used in our study. All specimens were frozen at -20°C shortly after removal. Prior to extraction, the material was coarsely minced and thoroughly washed in 0.5M sodium acetate at 4°C, sedimented, and resuspended in 0.5M acetic acid for three days to extract collagen. The supernatant was precipitated with 5% NaCl and centrifuged. The pellet was redissolved in 0.5M acetic acid and dialysed against 0.02M disodium phosphate. The precipitate formed was redissolved in 0.5M acetic acid and the collagen was purified by repeating the above process and finally lyophilized.⁹ The residue left after acid extraction was subjected to pepsin digestion (10-15 mg per g dry tissue weight). After 20 h, the suspension was centrifuged (70,000 g) for 1 h. The pellet was resuspended and digested again with pepsin twice. The supernatants were combined and subjected to sequential neutral salt precipitation.¹⁰ The pellet obtained with 0.7M NaCl was dissolved in 0.5 M acetic acid and dialysed against 0.05 M acetic acid and lyophilized. The salt-free lyophilized collagen was stored in a desiccator at 4°C.

**Segment-long-spacing (SLS) formation and electron microscopy**

Purified type I collagen was dissolved in 0.5M acetic acid, and the solutions were centrifuged at 100,000 g for 3 h to remove insoluble materials. To form SLS crystals, the solutions were dialysed against 0.2% ATP in 0.1M acetic acid at 4°C. The aggregates were taken on colloidon-coated grids, positively stained with 2% phosphotungstic acid, and examined under JEOL 1200EX-II electron microscope operating at 80 kV.¹¹

**Isolation and culture of dermal fibroblasts and measurement of growth and metabolic rate**

The portions of biopsied tissues collected in sterile culture media were washed thoroughly in sterile PBS (0.01M, pH 7.2) and treated with 0.5% trypsin 20 mM EDTA solution for 16 h at 4°C. After removal of the separated epidermis, the dermis was finely chopped and treated with 200 units/ml of collagenase in the presence of DME and 10% foetal bovine serum (FBS) at 37°C for 16 h. The digested tissue was centrifuged at 2000 rpm for 5 min and the pellet-containing cells were plated in a tissue culture flask containing a sufficient quantity of DME and 10% FBS. The plated cells were allowed to adhere and grow to confluence and were subcultured. In order to measure the growth rate, the fibroblasts were labelled, and ³H-thymidine was monitored at different time points. For the measurement of their metabolic activity, total protein content was measured at different time points.

**Results**

**Cases studied**

Scar samples were collected from patients admitted to the burns ward and the plastic surgery ward of two well-
known hospitals in Chennai, India, as described in the materials and methods section. The scar samples were collected and the patients were followed for a period of 20 years. During this period, 1223 patients with keloids and 1338 patients with hypertrophic scars were studied (Table I). Some of these scars were unsightly and led to difficulty or incapacity in the use of the affected organ (Fig. 1).

**Biochemical composition of ECM of scars**

The primary ECM components, namely water content, collagen, and proteoglycans, were analysed. Water content was estimated as the difference between the wet weight and dry weight of biopsied tissues. Collagen was estimated by the hydroxyproline content. Since GAGs are the major components of proteoglycans, the proteoglycan content of the biopsies was indirectly estimated by quantitation of GAGs. Scars showed higher amounts of all three components studied than normal skin. Among the scars, keloids showed a higher amount of these components (Table II).

**Table I - Cases studied from 1983 to 2003**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Keloids</th>
<th>Hypertrophic scars</th>
</tr>
</thead>
<tbody>
<tr>
<td>1223</td>
<td>1338</td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>257</td>
<td>Post traumatic</td>
</tr>
<tr>
<td>Post vaccineal</td>
<td>128</td>
<td>Post burn</td>
</tr>
<tr>
<td>Post traumatic</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>Post infective</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>Post burn</td>
<td>438</td>
<td></td>
</tr>
</tbody>
</table>

**Table II - Biochemical composition of scars**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Water content</th>
<th>Collagen</th>
<th>Glucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal skin</td>
<td>65%</td>
<td>50%</td>
<td>0.95%</td>
</tr>
<tr>
<td>Hypertrophic scar</td>
<td>70%</td>
<td>52.4%</td>
<td>1.8%</td>
</tr>
<tr>
<td>Keloid</td>
<td>75%</td>
<td>58.2%</td>
<td>2.2%</td>
</tr>
</tbody>
</table>

**Table III - Extractability of collagen from normal skin, hypertrophic scar and keloid**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Neutral salt sol.</th>
<th>6.5 M acetic acid</th>
<th>Pepsin treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal skin</td>
<td>5%</td>
<td>35%</td>
<td>60%</td>
</tr>
<tr>
<td>Hypertrophic scar</td>
<td>7%</td>
<td>40.5%</td>
<td>43%</td>
</tr>
<tr>
<td>Keloid</td>
<td>8.5%</td>
<td>48%</td>
<td>40%</td>
</tr>
</tbody>
</table>

**Table IV - Estimation of DNA synthesis and total protein content in dermal fibroblasts**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DNA synthesis (H-thymidine incorporation)</th>
<th>Estimation of total protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>24 hr</td>
<td>48 hr</td>
</tr>
<tr>
<td>Normal skin</td>
<td>213</td>
<td>319.5</td>
</tr>
<tr>
<td>Hypertrophic scar</td>
<td>247</td>
<td>395.6</td>
</tr>
<tr>
<td>Keloid</td>
<td>285</td>
<td>456</td>
</tr>
</tbody>
</table>

Figs. 1a,b - Post-burn hypertrophic scars. Fig. 1c - Post-burn keloid. Fig. 1d - Earlobe keloid.

Fig. 2 - Haematoxylin and eosin staining of paraffin-embedded sections of keloid (A), normal skin (B), and hypertrophic scar (C).
Thicker dermis in keloids with abnormal deposition of collagen

The histological assessment of scar and skin biopsies was performed by routine haematoxylin- and eosin-staining. Both keloid and hypertrophic scars showed a marked absence of epidermal ridges. A very thick epidermis was observed in hypertrophic scars, while in keloids a thick dermis was observed. Both types of scars showed heavy infiltration of cells in the dermal region (Fig. 2). More cells in the dermal region could contribute to greater deposition of ECM. The tissue sections were therefore subjected to electron microscopy in order to study collagen deposition in the ECM. Higher amounts of collagen deposition were observed in the ECM of both types of scars (Fig. 3). However, in keloid cases, collagen fibrillation appeared to be abnormal (Fig. 3a).

Higher amounts of acid-soluble collagen in keloid

The extractability of collagen in different types of buffer is used as a measure for identifying the extent of cross-linking in a given collagen sample. Collagen samples isolated from the skin and scar biopsies were extracted with neutral salt solution and 0.5 M acetic acid and by pepsin treatment. Normal skin biopsies showed higher amounts of pepsin-soluble collagen, while keloids showed higher amounts of acid-soluble collagen. Hypertrophic scars showed only slight variations in the amounts of acid- and pepsin-soluble collagen (Table III). A lower amount of pepsin-soluble collagen in keloids was an indication of lower levels of cross-linking in these samples. To confirm this, pepsin-soluble collagen from normal skin and keloids was subjected to SLS analysis by electron microscopy. This study clearly indicated that keloid collagen was less cross-linked than normal skin collagen (Fig. 4).

Keloid fibroblasts show more proliferation

Excess matrix deposition in cases of keloids and hypertrophic scars could be the result of a higher proliferation rate of dermal fibroblasts, of higher metabolic activity, or of a combination of both these events. Dermal fibroblasts were therefore isolated and propagated from the scar and skin biopsies. Keloid fibroblasts showed a higher proliferation than hypertrophic scars and normal skin fibroblasts (Graph 1). The rates of DNA synthesis and protein synthesis were studied in order to analyse the metabolic activity of these fibroblasts. Both these studies showed that keloid fibroblasts were metabolically more active than fibroblasts from hypertrophic scars and normal skin (Table IV). To test the hypothesis that a higher turnover of total proteins would lead to a higher turnover of secretory pro-
teins that would eventually result in more matrix deposition, we studied the cytoplasmic architecture using electron microscopy. An increased and intense staining for the endoplasmic reticulum (ER) was obtained in keloid fibroblasts (Fig. 5), thus confirming that keloid fibroblasts are indeed metabolically more active.

Discussion

This study was undertaken to help clinicians differentiate between the different types of abnormal scars that develop as the final result of trauma and surgical injuries. Clinical investigations were conducted over a period of twenty years in two major hospitals in the city of Chennai, India. Over a thousand samples of each scar type were studied during this period, and the patients’ progress was carefully followed and documented. The initial categorization of the scars was done by routine clinical procedures.

During such categorizations the medical community is posed with serious difficulties in the differentiation of the scars, and a comprehensive biochemical and ultrastructural analysis was therefore effected. The biopsied and operated scar specimens were first subjected to biochemical analysis of the major ECM components of the dermal region, namely collagen, water, and proteoglycans (Table II). All these components were found to be higher in keloids than hypertrophic scars and normal skin. The high water-content of the scar samples was indicated by magnetic resonance imaging of the patients (data not shown).

Patient follow-up revealed that the higher the water content of the scars, the longer it took them to regress. Excess water content could thus be one the causes for non-regression of keloids. Higher water content also indicates better extractability of ECM components. However, the easy extractability of ECM components also indicates poor matrix assembly. Excess water probably interferes with the fibrillar assembly of collagen. In normal skin the network-like assembly of collagen is maintained by the proteoglycans and GAGs. GAGs are responsible for the lateral assembly of collagen. Even though there is an excess amount of GAGs in scar tissues, the presence of excess water probably has an inhibitory effect on the lateral assembly of collagen, which is clearly seen in keloids (Fig. 3). Depending upon the association and assembly of collagen fibrils, collagen can be extracted in different types of buffers.

Conclusion

On the basis of this study it may be concluded that keloids and hypertrophic scars show distinct differences at the ultrastructural level. Excess matrix deposition, along with high water content, could lead to the abnormal assembly of collagen in keloids. Excess matrix deposition results from the high rate of proliferation and metabolic activity of dermal fibroblasts in keloids. Further research to probe the molecular mechanism underlying scar formation is necessary in order to design appropriate tools for the early detection of keloids and hypertrophic scars. Using these, it will be possible to develop stepwise strategies for suitable therapeutic interventions.

RÉSUMÉ. But : Les Auteurs se sont proposés d’évaluer les différences entre les cicatrices chéloïdes et hypertrophiques utilisant des techniques biochimiques et ultrastructurales. Méthode : Plus de 1000 patients atteints de divers types de cicatrices ont été étudiés et suivis pendant une période de 20 ans. Une analyse histochemique et biochimique a été effectuée pour ce qui concerne la composition de la matrice extracellulaire du derme. Au niveau ultrastructural, la déposition et l’assemblage du collagène ont été étudiés en utilisant la microscope électronique. Le taux de prolifération et l’activité métabolique des fibroblastes demeure isolés de la peau normale et des biopsies cicatricielles ont été étudiés pour évaluer la cause de l’excès de déposition de matrice dans les tissus cicatriciels. Résultats : L’évaluation des différents types de cicatrices a révélé que soit les chéloïdes soit les cicatrices hypertrophiques présentent un excès de déposition matricielle pour ce qui concerne le collagène et les protéoglycans. La chéloïde possède une haute quantité de collagène acide-soluble. En outre, l’assemblage des fibrilles de collagène est anormal aussi dans les ché-
loïdes. Les études sur la prolifération et l’activité métabolique ont démontré que les fibroblastes des chéloïdes présentent un taux de prolifération et d’activité métabolique plus élevé par rapport aux fibroblastes des cicatrices hypertrophiques et de la peau normale. Enfin, les fibroblastes des chéloïdes présentent une coloration élevée et intense pour ce qui concerne le réticulum endoplasmique, ce qui pourrait suggérer une justification plausible de l’activité élevée de ce type de fibroblaste. Conclusion : Les chéloïdes et les cicatrices hypertrophiques présentent des modèles ultrastructuraux très clairs soit de la déposition du collagène soit de son assemblage. Ces paramètres pourraient être raffinés avec des recherches ultérieures et ils pourraient donc constituer un instrument utile pour les chirurgiens qui doivent distinguer les différents types de cicatrices et adopter des stratégies thérapeutiques appropriées.

BIBLIOGRAPHY


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2006 TANNER-VANDEPUT-BOSWICK BURN PRIZE

The international Burn Foundation of the United States invites applications for the 2006 Tanner-Vandeput-Boswick Burn Prize to be awarded at the September 2006 ISBI Congress in Fortaleza, Brazil.

PURPOSE OF THE AWARD
The prize was conceived and established to promote the aims of the International Society for Burn Injuries and to motivate individual investigators to perform research, undertake patient care and treatment, and attempt to solve other aspects of the burn problem.

ELIGIBILITY
The prize will be awarded to a person or persons who have made an outstanding contribution to any aspect of burn management or to an outstanding practitioner of burn treatment. This could be a specific achievement or might represent a body of work over a period of years. The recipient does not have to be a member of the ISBI nor a physician, but be responsible for major advancement in the field of burn care.

THE AWARD
The prize consists of a gold pin and a cash payment estimated to be over $ 100,000.

NOMINATIONS
A candidate may make application on his own behalf or nominations may be submitted by colleagues.

DEADLINE FOR APPLICATIONS
Deadline for receipt of applications in Denver, Colorado will be February 28, 2006.

HOW TO APPLY
Nominations/applications must include:
• Completed application letter
• Letter of nomination
• One-page summary detailing research & accomplishments of applicant
• CV including list of publications
• Two additional letters of support from colleagues

FOR MORE INFORMATION AND APPLICATION MATERIALS
International Burn Foundation
P.O. Box 24386 - Denver, CO 80224 USA
intlburnfndn@yahoo.com - Phone 303.985.4065