A Wound Contraction Experimental Model for Studying Keloids and Wound-healing Modulators

*Fábio Kamamoto, *Andre Oliveira Paggiaro, †Andrea Rodas, *Marisa Roma Herson, †Monica Beatriz Mathor, and *Marcus Castro Ferreira

*Laboratório de Cirurgia Plástica, Faculdade de Medicina da Universidade de São Paulo; and †Centro de Tecnologia das Radiações, Instituto Pesquisas Energéticas e Nucleares da Universidade de São Paulo, São Paulo, Brazil

Abstract: Preventing and treating hypertrophic and keloid scars is difficult because of the lack of knowledge about their genesis. Tissue repair can be studied with biocompatible matrices and ex vivo cultures of different cell types. We used an experimental model where collagen gels populated by human fibroblasts underwent progressive contraction, allowing the study of wound healing remodeling. The fibroblast-populated lattices showed the greater contraction of the gel populated by fibroblasts from keloids versus fibroblasts from normal skin. Moreover, fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) involved in scar formation were added to the collagen gels populated by normal skin fibroblasts. TGF-β caused an increase in gel contraction; FGF did not. The mean percentages of contraction of the gels populated by keloid fibroblasts were very similar to the percentages of gels populated by normal skin fibroblasts with added TGF-β. These observations confirm the existing hypothesis that TGF-β may be involved in keloid formation. Key Words: Tissue engineering—Human fibroblasts—Collagen lattices—fibroblast growth factor—transforming growth factor beta—Keloid.

Wound healing is a complex phenomenon of vital importance to human beings. It encompasses an organized sequence of catabolic and anabolic events that depend on cellular and biochemical actions. The search for a better understanding of the wound healing process has been a constant in medicine. In the 16th century, Ambrose Pare discovered through his work that the use of rose oil could accelerate wound healing (1).

For descriptive purposes, the biological processes of wound healing can be divided into the following four phases: hemostasis, inflammation, proliferation, and remodeling (2). Although these processes are described as distinct phases, significant overlap occurs. The initial physiologic response to an injury is hemostasis. The clotting cascade is activated, and fibrin polymerization occurs as a mature clot is formed in conjunction with the aggregated platelets. Inflammation is carried out mainly by macrophages. Their importance lies in the secretion of multiple cytokines and growth factors that mediate various cellular and biochemical events responsible for healing. During the proliferative phase of healing, the migration and proliferation of several cell types primarily occur, leading directly to the repair of tissue integrity. As the fibroblasts and vascular endothelial cells migrate into the provisional matrix, they begin to proliferate and cellularity of the wound increases. The remodeling phase is the most important in plastic surgery, because it determines the final appearance of scars. This appearance depends mainly on two factors: the reorientation of collagen fibers and granulation tissue contraction (2). The growth factors fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) interfere with wound contraction. FGF inhibits the production of collagen by fibroblasts (3,4). TGF-β increases the expression of different kinds of collagen, and thus it may play a role in the formation of pathological scars, as keloids do (5,6). Scars frequently cause tightness, pain, and itching for the patient, and no efficient treatment exists for this pathology (7).

The mechanisms that ultimately lead to the formation of these pathologic scars and the availability of new investigative tools arising from tissue bio-
engineering have stimulated new studies about scar formation. Bell et al. in 1979 (8) described an in vitro model of collagen gel that decreases in diameter when populated with human fibroblasts. This model is considered adequate to simulate the contraction of the wound observed in vivo (9,10). Based on the concept proposed by Bell, our first objective was to implement a bioengineering model that mimics the contraction generated by fibroblasts and then to study this process with scanning electron microscopy. As a second step, we performed two independent complementary studies. First, we compared the contraction of collagen gels populated with normal skin fibroblasts with others populated with fibroblasts from keloids. Second, we analyzed the influence of the addition of FGF and TGF-β over collagen matrices populated with normal skin fibroblasts.

MATERIALS AND METHODS

Culture of human fibroblasts
Cutaneous surplus obtained from reduction mammoplasties performed in patients between 20 and 40 years old was used as raw material for isolating human fibroblasts from normal skin. The fibroblasts of keloids were isolated from scars excised during plastic surgery performed at the Hospital das Clínicas of the University of São Paulo.

The explant technique was used to isolate the fibroblasts (7). Culture medium (Dulbecco’s Modified Eagle Medium, DMEM; GIBCO, Carlsbad, CA, U.S.A.) plus 10% fetal calf serum (FCS; GIBCO) plus penicillin, 10,000 U/ml and streptomycin sulfate 10,000 g/ml (GIBCO) was changed every 3 days. After 2 weeks, we observed fibroblasts around the dermo-epiderm fragments. The fibroblasts multiplied up to semiconfluence. This amplification was performed with 0.05% trypsin until approximately 1 x 10⁶ fibroblasts was reached, the cell number used in each experiment.

Collagen solution preparation
The collagen used in the present work was obtained by an extraction technique with acetic acid of collagen type I of Wistar-Fur rat tail tendons (8). The collagen type I concentration was defined by hydroxy-proline measurement.

Collagen solution polymerization, inclusion of fibroblasts in the matrix
In 24 multiwell plates (FISHER 08-772-1, Pittsburgh, PA, U.S.A.), 1 million fibroblasts were included in 400 µl of phosphate buffer solution (PBS, 1x), 100 µl of 10x PBS, and 100 µl of 0.1 M NaOH, and finally, 400 µl of collagen type I solution. Once the gel polymerized, 2 ml of DMEM plus 10% FCS was added. The gel was gently unfastened from the culture well walls. Plates were kept in an incubator with 5% CO₂ at 37°C.

Macroscopic evaluation of the gel contraction
The area of collagen gel populated with fibroblasts was photographed with a digital camera (Sony, CYBERSHOT, P130 Tokyo, Japan). At 12, 24, 36, and 48 hr after the experiment had begun, the gel area was calculated with UTHSCSA Image Tool for Windows version 3.0 software (11).

The percentage of gel contraction in each interval studied was calculated with the following formula:

Percentage of contraction = (A1 – A2)/A1 x 100, where: A1 = initial gel area and A2 = area at the observed intervals.

Evaluation by electron scanning microscopy of the gel populated with normal skin fibroblasts
At each observation interval (i.e., 12, 24, 36, and 48 hr), samples of normal skin fibroblast-populated gels were cryoprotected with 40% dimethyl sulfoxide solution (40% DMSO), and frozen at -80°C. Later, samples were lyophilized, fixed, and covered with gold. Finally, they were analyzed with an electron-scanning microscope (Phillips XL30, Groenewoudseweg, Eindhoven, The Netherlands).

Comparison between normal skin fibroblast-populated gels and keloid matrices
Nine collagen gels populated with normal skin fibroblasts and another nine with keloid cells were photographed every 12 hr and observed for 48 hr.

Comparison of normal skin fibroblast gels with TGF-β and FGF
Study groups comprised nine collagen matrices composed of normal skin fibroblasts (control), nine with normal fibroblasts plus 50 ng/ml FGF (group FGF), and another nine with normal skin fibroblasts and 10 ng/ml TGF-β (group TGF-β). The three groups were also photographed every 12 hr and observed for 48 hr. At each study interval, contraction percentages were calculated. Later, those areas were compared by statistical analysis for repeated measurements.

Statistical analysis
The gel’s contraction data were analyzed with ANOVA and are expressed as mean percentage of contraction and standard deviation.
**RESULTS**

**Evaluation by electron scanning microscopy of the gel populated with fibroblasts from normal skin**

After 12 hr, we noticed in the analyzed samples low cellular density with collagen fibrils randomly arranged (Fig. 1A). At 24 hr, the fibrils became dense, like collagen fibers (Fig. 1B). At 36 hr, a thickening process occurred in the fibers, resulting in greater parallelism; an increase in fibroblast number per observed field (Fig. 2A) was also noticeable. Finally at 48 hr, we noticed the maximum thickness of the fibers as well as the highest cellular density (Fig. 2B).

**Comparison between matrices populated with normal skin fibroblasts and keloids**

We did not observe any contraction in the gels without cells.

The gels populated with fibroblasts from keloid scars underwent progressive contraction throughout the time interval studied until 48 hr. After that, we did not see progressive diminution of the gel area. We noted a greater contraction of the gels populated with fibroblasts from keloids per time studied in relation to the gels populated with fibroblasts from normal skin ($P = 0.0009$). The values related to the contraction percentage of gels populated with normal skin and keloid fibroblasts during the experiment can be observed in Table 1.

The reduction percentage of the original area of the gels generated by the fibroblasts from keloids in relation to normal skin and without cells can be observed in Figure 3.

**Comparison between matrices populated with normal skin fibroblasts with TGFβ and FGF**

We noticed greater contraction in the gels populated with fibroblasts from normal skin with the addition of FGF when compared with that in the gel with cells only. Nevertheless, these data are not statisti-
cally significant \( (P = 0.14) \). On the other hand, in the gels with added TGF-β, faster contraction was noted, which was approximately 15% faster than that in those with no modulating substances \( (P = 0.0002) \).

Table 1 shows the reduction percentages of the gel area in relation to the initial area in each group.

Figure 3 shows the curves of contraction percentage (average) of the gels after the addition of FGF and TGF-β.

**Comparison between the contraction of matrices populated with keloid fibroblasts and normal skin fibroblasts with TGF-β**

We noticed a similarity in the contraction curves of the gels populated with normal skin fibroblasts with addition of TGF-β and with keloid fibroblasts \( (P = 0.749) \). Figure 3 allows comparison of the contraction percentage curves (average values) of the gels with normal skin fibroblasts and normal skin fibroblasts after the addition of TGF-β and keloid fibroblasts.

**DISCUSSION**

Advances in biotechnology can improve the understanding of the phenomenon involved in anesthetic and pathologic scar formation, allowing the management of growth and function of the cell, and also the exploration of new tools for the prevention and treatment of hypertrophic and keloid scars. The model described by Bell et al. (8) has been accepted in the literature as adequate for the study of wound contraction, because it includes the two fundamental dermal participants in scar formation: the extracellular matrix and fibroblasts. Consequently, based on Bell’s descriptions, we established in our medium a collagen gel model, which underwent contraction after fibroblast incorporation. The collagen type I gel with no addition of fibroblasts did not undergo measurable contraction during the study. Thus, it is possible to state that the collagen gel does not have an intrinsic ability to contract. On the other hand, we can observe that the gels populated with 10⁶ human fibroblasts underwent a reduction in diameter. So, we can conclude that gel contraction is due to the interaction between fibroblasts and the collagen fibrils around them.

The macroscopic contraction of the collagen matrices with normal skin fibroblasts began in the first 12 hr and showed progression at the intervals, reaching their maximum in 48 hr. After this interval, we did not observe any measurable contraction. One possible explanation for this might be the “saturation” of the fiber contraction mechanisms, meaning that, after that period, the fibers are already in their maximum organizational level.

In the scanning electron microscopy study, we observed that the collagen matrices after 12 hr had collagen fibers randomly dispersed around the fibroblasts. However, after 24 hr, those fibers started to undergo a reorganization process, which was more evident after 36 hr. The fibrils began to form sheaves, and the space once occupied by fibers distributed randomly began to show pores among the cells. Finally, after 48 hr, the contraction reached the

---

**TABLE 1. Average values of the contraction percentages of the gel populated with fibroblasts from normal skin and keloids and the gel populated with fibroblasts from normal skin with addition of FGF and TGF-β**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Average of gel diminished area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hr</td>
</tr>
<tr>
<td>Normal skin (n = 9)</td>
<td>50.67 ± 9.34</td>
</tr>
<tr>
<td>Keloid (n = 9)</td>
<td>66.30 ± 9.7</td>
</tr>
<tr>
<td>Without cells (n = 9)</td>
<td>0</td>
</tr>
<tr>
<td>TGF-β (n = 9)</td>
<td>58.32 ± 8.68</td>
</tr>
<tr>
<td>FGF (n = 9)</td>
<td>52.21 ± 6.96</td>
</tr>
</tbody>
</table>

*Artif Organs, Vol. 27, No. 8, 2003*
maximum of fiber organization and possible cellular concentration.

We can correlate these increases in cellular concentration and collagen fiber organization with the macroscopic gel contraction. Possibly, the gel contraction is due to the orientation and reorganization of collagen fibers, similar to that which happens with cotton. In its raw state, cotton wool also has fibers randomly dispersed and with low mechanical resistance. However, after being spun into thread, it undergoes a decrease in area and an increase in mechanical resistance.

In the study where we compared the contraction of gels populated with fibroblasts from normal skin with those populated with keloid cells, we observed a faster contraction that was, on average, 15% faster in gels populated with keloid fibroblasts versus those populated with normal skin. This strongly suggests that the keloid fibroblasts have a greater ability to cause contraction when they interact with the surrounding extracellular matrix. Perhaps this higher contracting ability is involved in the formation of increased anesthetic scars. When FGF was added to the collagen matrices with normal skin fibroblasts, a contraction was generated that was ~10% greater than contractions in the gels that did not receive this substance. These data are not statistically significant ($P = 0.14$).

The TGFβ-generated contraction was more accelerated and, on average, 15% greater than that in the gels without growth factor ($P = 0.0002$), showing the great sensitivity of the fibroblasts to these substances and increasing the possibility of using them in clinical treatment to accelerate wound contraction and healing (12,13).

The comparison of the contraction of normal skin fibroblast-populated gels with the addition of TGF-β and those with keloid fibroblasts showed a great similarity between them. Perhaps this similarity observed in the two graphics is not a coincidence; we can speculate from these data that those patients with a propensity to form keloid scars may have fibroblasts that have been exposed more to the presence of TGF-β. Liu et al. (14) corroborated this fact by observing a higher expression of the gene responsible for the profibrotic agent TGF-β in fibroblasts of collagen gels exposed to mechanical stress. Mikulecc and Hansono (15) detected a greater production of TGF-β in cultures of fibroblasts from keloid scars when compared with cultures of normal skin fibroblasts. We hope our findings increase understanding about a phenomenon involved in the genesis of pathologic scars, and when these data are applied in the clinical field, new therapeutic options may arise.

Acknowledgments: We thank Instituto de Pesquisas Nucleares da Universidade de Sao Paulo (IPEN), scanning electron microscopy; Fundação de Amparo a Pesquisa (FAPESP) for financial support.

REFERENCES