Scanning Electron Microscopy as a Tool for the Evaluation of Collagen Lattices

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Abstract

An important component of tissue healing and wound contraction is the re-arrangement of ground collagen fibers, which can ultimately influence the final quality of scars. Using the experimental model suggested by Bell in 1979 of *in vitro* contracting collagen gels seeded with human fibroblasts. The analysis of the changing macroscopic gel areas by the observation of the tri-dimensional distribution of collagen fibers can be done employing scanning electron microscopy (SEM). Progressive macroscopic decrease in the collagen gels areas could be measured throughout the experiments. Scanning electronic microscopy performed at 12 hours disclosed low cellular densities and the random disposition of collagen fibers. After 24 hours, collagen fibers showed some re-arrangement, their orientation possibly determined by nearby fibroblasts. At 36 hours, improved orientation of fibers resulted in an increase in thickness, and higher cellular density could be noted. At 48 hours maximal fiber thickness and fibroblast densities were observed .

Keywords: Collagen Lattice; human fibroblasts; lattice contraction, collagen fibers; SEM.

Introduction

Wound contraction is an essential step for the closure of healing tissue as well as a major contributor to the quality of the final scar. This contractile process is thought to be caused through the action of mesenchymal cells, called fibroblasts. These cells are attracted by inflammatory mediators to the wound in a second phase of scaring, to produce collagen type I and to interact with the surrounding collagen matrix, causing reorganization of this structure. This phenomenon results in the contraction of the wound.

In 1979, Bell *et al* (1) described an *in vitro* model where fibroblasts seeded in a collagen gel caused a macroscopically measurable contraction of this matrix. This method allows the study of cell contractile properties in an in vivo-like environment through tissue engineering of human wound-healing equivalents.

The mechanism by which fibroblasts induce gel contraction is not known. Some works (2,4) demonstrate that the interaction of the surrounding collagen fibers and the fibroblasts generates forces causing a dynamic process that reorganize the system, producing wound's contraction.

Thus, to study the functional aspects of dermal and wound-healing fibroblasts, Bell's model was used in this experimental work. The authors relate the observed macroscopic changes in the collagen lattices areas with the microscopic tri-dimensional re-arrangement of collagen fibers observed in scanning electron microscopy of the preparations.

Materials and Methods

Cell culture

Human fibroblasts were obteined from foreskin specimens obtained from Hospital das Clinicas de São Paulo. The specimen were freed of subcutaneous tissue and the small pieces (approximate 1 mm^3) were placed in 35 mm plastic tissue dishes. The cells were grown in Dulbecco's Modified Eagle's Medium, supplemented with SFB 10% in an incubator gassed with 5% CO_2 air at 37°C. Cells were grown to confluence and amplified and they were used in their fifth passage.

Collagen solution

Type I collagen solutions were obtained from rat tail tendons immersed in acetic acid 0,1 M. This collagen (2,5 mg/ml) was stored at 4°C until use.

Lattice

Initially, $1x10^6$ human fibroblasts were mixed with 400 µg of PBS (1X), after this 400 µg of murine collagen type I, plus 100 µg of NaOH (0,1N) and 100 µg of PBS (10X) were added to prepare the collagen matrices. They were gently freed from the walls of the culture dishes after 3 hours. The areas of the collagen gels were measured at 12, 24, 36, and 48 hours of the experiments with UTHSCSA ImageTool Softwear (5).
Collagen gel contraction was determined by the following formula:

$$\text{Percentage contraction} = \frac{A1 - A2}{A1} \times 100$$

Where A1 = initial area of gel
A2 = area at the observed interval.

Scanning Electron Microscopy

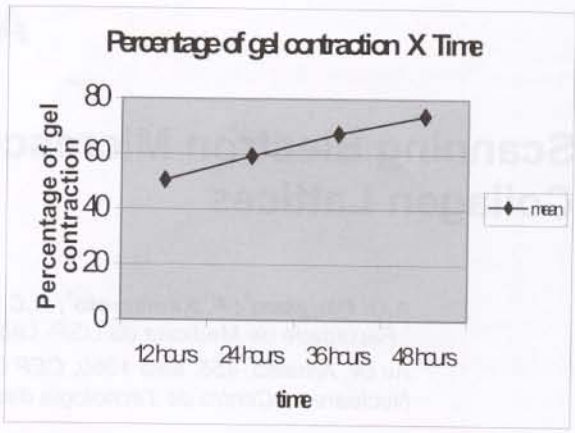
At each observation (i.e. at 12, 24, 36 and 48 hs) gel samples were crioprotected with DMSO 40%, and deep-frozen at –80°C. Later, they were liophilized, fixated, Spurr-coated and gold stained, and finally were scanned in a Phillips XL30 SEM.

Results

Collagen gel measurements are shown in Table 1, demonstrating a progressive decrease in initial size, as observed by a decrease of 74% of the original gel areas.

	12 hs	*24 hs*	*36 hs*	*48 hs*
Mean	50,67	59,40	67,50	74,03
Desvio	9,34	8,95	6,44	7,5
N	9	9	9	9

Table1. Percentage of gel contraction X Time



Grafic 1. The percentage of contraction of collagen lattice with 1 x 10^6 human fibroblasts

When the lattices were studied by scanning electron microscopy, we observed that at 12 hours they disclosed low cellular densities and a random disposition of collagen fibers (Figure 1). After 24 hours, collagen fibers showed some re-arrangement, their orientation possibly determined by nearby fibroblasts (Figure 2). At 36 hours, improved orientation of fibers resulted in an increase in thickness, and higher cellular density could be noted (Figure 3). At 48 hours maximal fiber thickness and fibroblast densities were observed (Figure 4).

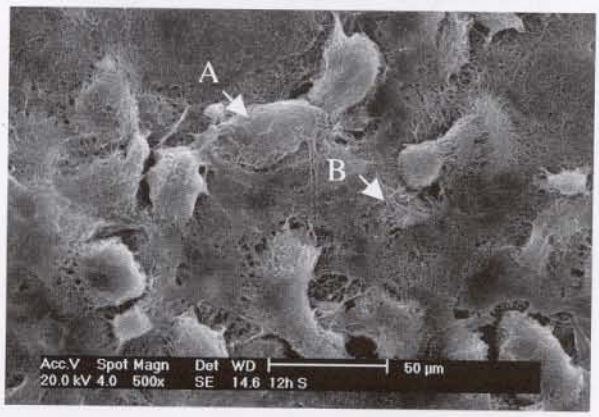


Figure 1. Surface of collagen gel after 12 hours of contraction with human fibroblasts (HF 151). X 500 . (A) fibroblast; (B) fiber collagen

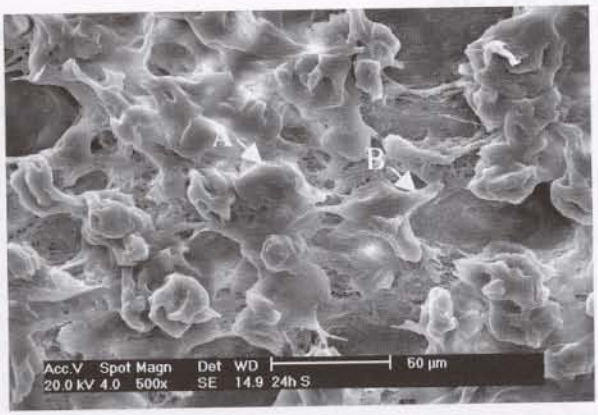


Figure 2. Surface of collagen gel after 24 hours of contraction with human fibroblasts (HF 151). X 500 . (A) fibroblast; (B) fiber collagen

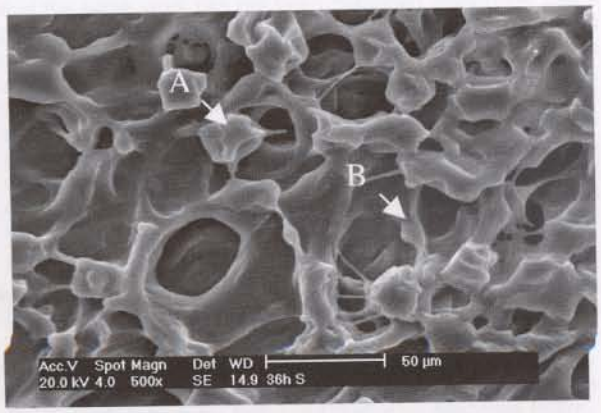


Figure 3. Surface of collagen gel after 36 hours of contraction with human fibroblasts (HF 151). X 500. (A) fibroblast; (B) fiber collagen

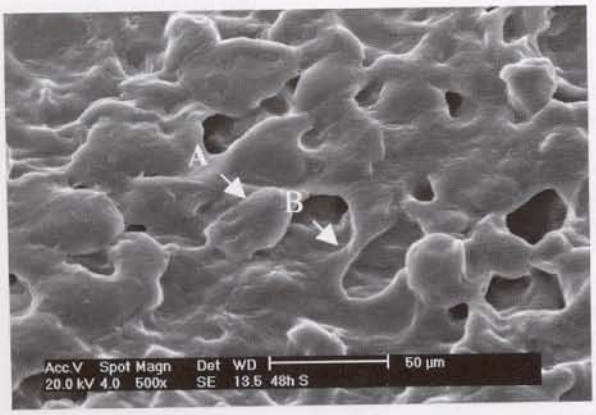


Figure 4. Surface of collagen gel after 48 hours of contraction with human fibroblasts (HF 151). X 500. (A) fibroblast; (B) fiber collagen

Discussion

An important component of tissue healing and wound contraction is the re-arrangement of ground collagen fibers, which can ultimately influence the final quality of scars. The mechanism of tissue contraction during wound healing is not completely understood. There are two theories proposed to explain this process. The first theory suggests that the contraction forces are generated by myofibroblasts and than are transmited to other cells and surrounding connective tissue. Thus this hypothesis suggests that the myofibroblasts would act as a multicelular unit to contract the tissue (6). The second theory proposes that the contraction is caused by the re-arrangement of collagen fibers through the action of fibroblasts. The dislocation forces created by these cells within the connective tissue would lead to the re-orientation of collagen fibers into thicker bundles as well as their contraction, as viewed in SEM by the re-arrangement and increased densities of the fiber bundles (2). *Berry et al.* (3) observed that 88% of the healing of a sacral wound occurred due to contraction, and the other 22% occurred due to scar formation. *Allen et al.* (4) observed in electron micrographs fibroblasts "adhering" to surrounding collagen fibers.

Different methods have already been used to study the contraction of collagen gels. Imunohysto chemic for *example is useful to study the proportion of* myofibroblasts containing α-smooth muscle actin in their cytoplasm compared to fibroblasts (8). Transmission Electron Microscopy gives the opportunity to study the interaction between the fibroblasts and the surrounding collagen fibers (4).

In this study the authors confirm these previous observations, relating the macroscopic changes observed in the collagen gels with the spatial re-arrangement and increase of thickness of collagen fibers as well as an increase in fibroblast density. The use of scanning electron microscopy to appreciate this three-dimentional phenomenon, proved to be a valuable tool for better understanding of the mechanisms involved in wound contraction and scar formation.

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