Long-Term Culture of Fibroblasts in Contracted Collagen Gels: Effects on Cell Growth and Biosynthetic Activity

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The purpose of these studies was to analyze the consequences of long-term collagen gel contraction on fibroblast growth and metabolic activity. After 4 weeks, floating gels were 98% contracted, and attached gels were 94% contracted. During this culture period, fibroblasts in floating gels regressed significantly compared to fibroblasts in attached gels, although the cells remaining in the floating gels were viable. In attached gels, fibroblasts were bipolar; whereas in floating gels, fibroblasts were stellate. Therefore, differences between survival of fibroblasts in attached and floating collagen gels might depend on cell shape. Similarly, extracellular matrix organization and its influence on cell shape might control fibroblast proliferation in granulation tissue. During long-term culture of fibroblasts in contracted collagen gels, 70%–80% of the starting collagen was degraded. Collagen synthesized by cells in 4-d cultures was mostly procollagen secreted into the medium. On the other hand, collagen synthesized in 4-week cultures was processed to α(I) chains and incorporated into the matrix. There also were other differences between the proteins synthesized by fibroblasts after short-term and long-term culture in contracted gels. These findings show that fibroblasts in long-term collagen gel cultures express unique growth and biosynthetic characteristics. J Invest Dermatol 93:792–798, 1989

During connective tissue repair, fibroblasts exhibit several different activities. At first, they migrate from adjacent tissues into the wound region. In the wound region, they proliferate and synthesize a collagen-rich extracellular matrix that fills the wound defect. The fibroblasts also contribute to the remodeling of newly synthesized extracellular matrix. Finally, the expanded fibroblast population regresses [1, 2]. Depending on the size and location of the wound, contraction of granulation tissue plays a role in wound healing [1, 2]. A subpopulation of wound fibroblasts called myofibroblasts may participate in the wound contraction phenomenon [3, 4].

To learn more about the regulation of fibroblast function during wound repair, we focused our studies on fibroblasts cultured in collagen gels [5]. This model is of particular interest because the cells rapidly reorganize and contract the collagen gels [6–8], analogous to wound contraction in vivo [9, 10]. Reorganization of collagen gels by fibroblasts requires cell motile forces [11] propagated throughout the interconnected collagen fibril network. Disruption of the interconnected network inhibits contraction [12]. During the initial phase of contraction, cells mechanically hold the reorganized collagen fibrils in place. Later, interstitial collagen bonds stabilize the reorganized collagen fibrils without cells [13].

Substantial differences in collagen gel contraction by fibroblasts occur, depending on geometrical constraints. Contraction of gels attached to underlying culture dishes results in decreased gel thickness without a change in gel diameter [7, 14]. Contraction of floating gels results in decreased gel thickness and diameter [6]. During contraction, collagen fibrils in attached collagen gels become aligned in the plane of cell spreading, and fibroblasts spread with a bipolar morphology. In floating collagen gels, on the other hand, collagen fibrils remain unorganized, and fibroblasts spread with a stellate morphology [15]. DNA synthesis by cells in floating gels decreases significantly after gel contraction [16, 17], unlike DNA synthesis in contracted, attached collagen gels [15]. Therefore, in collagen gels contracted for short periods (1–4 d), extracellular matrix organization and cell shape regulate fibroblast growth rather than gel contraction per se.

As described above, expression of different fibroblast functions during wound repair usually occurs during a period of weeks rather than days [1, 2]. Therefore, we investigated the long-term, in vitro consequences of collagen gel contraction by fibroblasts. We found that during 4 weeks in contracted, floating collagen gels, the fibroblast population regressed 90%. In parallel experiments, the fibroblast population in contracted, attached collagen gels expanded. Several activities not usually observed in short-term cultures, such as collagenase activity and procollagen processing, were evident during the long-term cultures. Details of these observations follow.

MATERIALS AND METHODS

Cell Culture in Hydrated Collagen Gels Human skin fibroblast monolayer cultures were established from foreskins obtained at circumcisions and maintained and harvested as before [20]. Preparation of hydrated collagen gels from Vitrogen “100” collagen (Collagen Corp, Palo Alto, CA) has been described previously [12, 13]. Fibroblasts were added to the neutralized collagen solutions (1.5 mg/ml) at a concentration of 10⁶ cells/0.2 ml. Aliquots (0.2 ml) of the cell/collagen mixtures were warmed to room temperature and placed in Costar 24-well culture plates. Each aliquot occupied an area outlined by a 12-mm-diameter circular score within a well.
Gels were polymerized by raising the temperature to 37°C. The samples were incubated for 60 min, after which 1.0 ml of culture medium [Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml ascorbic acid] was added to each well. After polymerization, the fibroblasts were dispersed throughout the gels. To obtain cultures of floating collagen gels, the attached gels were gently lifted off the bottom of the wells with a spatula.

**Measurement of Gel Contraction** Gel contraction was determined by measuring gel volume [15,17]. We added 1 μCi of [3H-H2O (1 mCi/g, New England Nuclear) to the culture medium. After 1 h, the medium was removed, and the gels were rinsed quickly and dissolved in 0.5 ml of 1M NaOH. The samples were neutralized with HCl, mixed with 10 ml Budget Solve (RPI Corp.), and counted in a Beckman Scintillation spectrometer. Initial gel volume was determined in gels without fibroblasts.

**Microscopy** Microscopic analysis was done as described previously [8]. Samples were fixed for 2 h at 4°C with 2% glutaraldehyde, 1% paraformaldehyde, and 1% tannic acid, in 0.1M cacodylate (pH 7.4), postfixed for 30 min at 22°C with 1% aqueous OsO4, and stained en bloc with 1% aqueous uranyl acetate for 30 min at 4°C. After dehydration, specimens were embedded in Epon 812. Thick sections were stained with 1% toluidine blue and examined and photographed using a Zeiss Photomicroscope III. Thin sections were examined and photographed with a Philips 300 electron microscope.

For attached gels, multiple thick sections from central and marginal regions of the gels were prepared and examined. No differences in cell morphology were evident in the different regions. For floating gels, thick sections contained both the margins and central region of the gels. The thick sections photographed were selected based on their typical appearance, and thin sections were prepared from the same block faces.

**Cell Recovery** Collagen gels were solubilized by incubation for 140 min at 37°C with 0.2 ml of 150 mM NaCl, 10 mM Ca acetate, and 20 mM HEPES (pH 7.2), containing 2 mg/ml collagenase (Type I, Sigma Chemical Co.). Subsequently, 0.1 ml of 0.25% trypsin ( Gibbico) and 0.02 ml 0.3M EDTA were added to the samples and incubated for an additional 20 min at 37°C. This combination of treatments dissolved the gels and dissociated any cell clumps. Aliquots of the samples were mixed with trypsin blue, and cell number was measured with a hemocytometer.

**Collagen Synthesis** Collagen synthesis was measured by the proline-free collagenase method [18]. Cultures were radiolabeled metabolically for 24 h with 10 μCi/ml [3H-L-proline (126.9 Ci/m mole, New England Nuclear) added in fresh culture medium. At the end of the incubations, the medium was removed. The gels were dissolved by incubating them for 90 min at 37°C with 0.4 ml of 10 mM HCl, and then the solubilized gels were neutralized with NaOH. One-half of each medium and solubilized gel sample was brought to 1 ml containing 30 mM Tris, 6 mM Ca acetate, pH 7.2, and treated with collagenase (5 BTC units/ml, Form III, Advanced Biofactures Corp) for 3 h at 37°C. Bovine serum albumin (0.5 mg/ml) was added to collagenase-treated and untreated samples to act as carrier protein, after which all the samples were subjected to three precipitations with 10% TCA at 4°C. The final precipitates were dissolved with Protosol, and radioactivity was measured as above.

Cultures to be analyzed by SDS-PAGE were radiolabeled metabolically for 4 d with 30 μCi/ml [35S-methionine (1072 Ci/m mole, New England Nuclear) added in methionine-deficient culture medium. At the end of the incubations, the medium was removed, and the gels were solubilized as above. Medium and gel samples were treated with or without collagenase Form III and TCA precipitated as above.

**Collagen Degradation** Radiolabeled collagen was prepared by acetylation with [3H-acetic anhydride (2.5 mCi, 50 mCi/m mole, New England Nuclear Corp), as described [19]. The specific radioactivity of the radiolabeled collagen preparation was 1.5 × 10⁶ cpm/mg. Gels containing radiolabeled collagen and fibroblasts were polymerized as described above for non-radiolabeled collagen. At the times when collagen degradation was measured, the culture medium was removed from the incubations. The gels were rinsed briefly and dissolved with 1M NaOH, neutralized with 1M HCl, and radioactivity was counted with a scintillation spectrophotometer as above.

**Electrophoresis and Fluorography** Samples for SDS-PAGE were dissolved in reducing sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% mercaptoethanol, pH 6.8). Electrophoresis was performed using 4%–16% acrylamide gradient slab gels containing a gradient of 3–8 M urea [20]. Acryl-Aide (FMC Corp) was used as the crosslinker, and gels were supported on GelBond-PAG film (FMC Corp). After electrophoresis, gels were fixed and stained with Coomassie blue. Gels were impregnated for 1.5 h at 22°C with En3Hance (New England Nuclear Corp) containing 2% glycerol. Gel films were dried in a 60°C oven and exposed to Kodak XAR-5 film on a Cronex intensifying screen (E.I. Dupont De Nemours & Co.).
RESULTS

Contraction of Collagen Gels During 4 weeks of Culture
After 2 d of culture, floating collagen gels containing fibroblasts contracted to a slightly greater extent than attached collagen gels (~90% vs 80%) (Fig 1). After 4 weeks, the extent of contraction of the floating gels and attached gels was 98% and 94%, respectively. Therefore, the volume of attached gels was about 3 times greater than the volume of floating gels. Non-specific contraction, i.e., decrease in gel volume without cells, was ~10% after 2 weeks and ~20% after 4 weeks.

Collagen gel contraction could have occurred in part because of collagen degradation. There was only a slight loss of collagen from the gels during the initial 1–2 d of culture (Fig 2). After 1 week of culture, however, collagen degradation became quite noticeable. By the end of 4 weeks, ~80% of the starting collagen was degraded in the attached gels and ~70% in the floating gels (Fig 2). Considering the differences in gel volume (Fig 1) and collagen degradation (Fig 2), it could be calculated that after 4 weeks the collagen density (1.5 mg/ml in the original gels) was about 20 mg/ml in the floating gels and 4.5 mg/ml in the attached gels. These values ignore new collagen synthesis. By way of comparison, the collagen concentration in skin exceeds 50 mg/ml.

Some collagen loss from the gels also occurred without added fibroblasts (~30% during the first 2 weeks of culture). This non-specific loss did not increase further during the first 2 weeks of culture. Therefore, a subtraction of the collagen apparently incorporated into the gels in an unstable manner. The non-specific decrease in gel volume (Fig 1) could be accounted for by the non-specific loss of collagen from the gels (Fig 2). Based on cell recovery (see Fig 5 below), collagen loss normalized on a per cell basis was much higher in floating gels than in attached gels.
Morphologic Features of Collagen Gels contracted for up to 4 Weeks Fibroblasts in attached collagen gels after 24 h of contraction were bipolar cells, elongated parallel to the culture dish surface (Fig 3A). In contracted, floating gels, fibroblasts had a stellate appearance and random arrangement (Fig 3B). The bipolar morphology of fibroblasts in attached collagen gels was similar after 2 weeks (Figs 3C and 4A) and 4 weeks (Figs 3E and 4C) of culture. During this time, the cells became more closely packed together. In floating collagen gels, distribution of fibroblasts was unchanged after 2 (Figs 3D and 4B) and 4 weeks (Figs 3F and 4D) of culture. Cell morphology remained stellate, but the cell pseudopodia became more prominent relative to the cell bodies.

In attached collagen gels, the extracellular matrix developed an uneven distribution during long-term culture (Fig 3E). Also, a significant decrease in collagen fibrils visible by electron microscopy occurred between 2 and 4 weeks (compare Fig 4C and A). In the floating collagen gels, the density of extracellular matrix and distribution of collagen fibrils did not change noticeably between 2 (Figs 3D and 4B) and 4 weeks (Figs 3F and 4D).

Cell Recovery from Contracted Collagen Gels Although floating collagen gels contracted more than attached collagen gels, the density of fibroblasts in attached gels appeared greater than in floating gels (Fig 3). This observation was quantified by dissolving the gels and counting the cells that could be recovered. In the experiment described in Fig 5, attached gels were contracted by fibroblasts for 1 d and subsequently cultured either attached to dishes or floating in medium. Between 2 and 14 d, cells in attached gels underwent at least two doublings to \( \sim 4.8 \times 10^5 / \text{gel} \). During the same period, cells in floating gels decreased by \( > 50\% \) to \( \sim 0.35 \times 10^5 / \text{gel} \). Although there was no further increase in cell number in attached gels between 2 and 4 weeks, the cells in floating gels decreased by another 50% during that period.

To learn if the fibroblasts in collagen gels cultured for 4 weeks were viable, cells harvested from the gels were re-plated in tissue culture dishes. Most of the cells from attached gels were viable and started to divide after a 1-d lag period (Fig 6). Only about one-half the cells from the floating gels were viable. These cells started to divide after 1 d at the same rate as the cells harvested from attached gels.

Collagen Synthesis in Contracted Collagen Gels Figure 7 shows the rate of collagen synthesis, measured by incorporation of radiolabeled proline into collagenase-sensitive extracellular matrix and medium proteins, at several times during the 4-week culture period. Consistent with the cell recovery data, there was a signifi-
cant increase in collagen synthesis in attached gels and a decrease in collagen synthesis in floating gels. Normalized by cell number, collagen synthesis in 4-week attached gels was twice as high as collagen synthesis in 4-week floating gels.

SDS-PAGE profiles of collagens and other proteins synthesized by fibroblasts differed for 5-d and 4-week attached gel cultures. We focused our attention on attached gels because cell recovery from the floating gel cultures was so low. Fibroblasts in the attached gels were radiolabeled with 35S-methionine. Medium and gel samples from 5-d cultures (Fig 8, lanes 1 and 3, respectively) contained 10–20 radiolabeled polypeptides, most of which were insensitive to collagenase (Fig 8, lanes 2 and 4). Two major collagenase-sensitive components around 180 and 150 kDa and a collagenase-insensitive component at 220 kDa were identified previously as procollagens α1(I) and α2(I) and fibronectin [21,22]. These three components were the major polypeptides found in medium from fibroblast monolayer cultures radiolabeled with proline. After overnight pepsin treatment in the cold, the 180- and 150-kDa components shifted to molecular masses 145 and 122 kDa [α1(I)] and α2(I) collagen chains], and the 220-kDa component disappeared (data not shown).

In contrast to the 5-d samples, cellular processing of procollagen to collagen α chains occurred in gels contracted for 4 weeks (Fig 8, lanes 7 and 8). Besides the changes in collagen processing, there were several other major changes in proteins synthesized by fibroblasts in 5-d and 4-week cultures. A prominent polypeptide of ~65 kDa appeared in the medium samples from 5-d cultures (lanes 1 and 2, asterisk) but not in medium samples from 4-week cultures (lanes 5 and 6). On the other hand, a pair of peptides ~116 kDa were

![Figure 5](image5.png)

**Figure 5.** Recovery of cells from contracted gels. Collagen gels containing fibroblasts were attached to culture dishes for 1 d and then cultured further, either attached or floating in culture medium. At the times shown, the gels were dissolved and the number of recovered cells determined. Data are the averages ± S.D. from duplicate samples. Other details are in Materials and Methods.

![Figure 6](image6.png)

**Figure 6.** Growth of cells recovered from 4-week contracted gels. Collagen gels were attached to culture dishes or floating in culture medium for 4 weeks. Then the gels were dissolved, and 10^6 recovered cells were plated in culture flasks in growth medium. At the times shown, the cells were harvested and cell number was determined. Data are the averages ± S.D. from duplicate samples. Other details are in Materials and Methods.

![Figure 7](image7.png)

**Figure 7.** Collagen synthesis in contracted gels. Collagen gels containing fibroblasts were attached to culture dishes for 1 d and then cultured further, either attached or floating in culture medium. Cultures were incubated with radiolabeled proline for 24 h prior to the times shown, and incorporation of radioactivity into collagen was measured. Data are the averages ± S.D. from triplicate samples. Other details are in Materials and Methods.

![Figure 8](image8.png)

**Figure 8.** Pattern of collagen and protein synthesis in attached collagen gel cultures. Fibroblasts in attached collagen gel cultures were radiolabeled with 35S-methionine between 1 and 5 d of culture or 24 and 28 d of culture. Lanes 1–4: 5-d cultures; Lanes 5–8: 28-d cultures. Samples were medium (lanes 1 and 5); medium + collagenase (lanes 2 and 6); gel (lanes 3 and 7); and gel + collagenase (lanes 4 and 8). Five-day cultures contained procollagens but not collagen α chains, but collagen α chains were a major component in the gel from 28-d cultures. Most fibronectin (Fn) was found in the culture medium, not in the gels. A major ~66-kDa component (asterisk) in the medium from 5-d cultures was not observed in 28-d cultures. A major ~116-kDa pair of components (asterisk) in the medium from 28-d cultures was not observed in the 5-d cultures. Pooled samples from two culture wells were loaded onto each lane. Other details are in Materials and Methods.
barely visible in medium from 5-d cultures (Lanes 1 and 2, asterisk) but were major constituents in 4-week cultures. Also striking was the greater accumulation of FN in the medium samples compared to the gel samples. Apparently, most of the FN synthesized within contracted gels did not incorporate into the extracellular matrix.

DISCUSSION

The purpose of these studies was to analyze the consequences of long-term collagen gel contraction on fibroblast growth and metabolic activity. Fibroblasts in attached gels and in floating gels differed significantly in their responses to long-term contraction as summarized in Table I. After 4 weeks, floating gels were 98% contracted and attached gels were 94% contracted. During this culture period, fibroblasts in floating gels regressed ~ fivefold, while fibroblasts in attached gels increased ~ fivefold. The cells remaining in the floating gels were viable, however. When returned to routine culture conditions, they divided at the same rate as cells removed from attached collagen gels.

Previous studies showed that DNA synthesis by fibroblasts in floating collagen gels decreased after gel contraction [16,17], but a similar decrease did not occur after contraction of attached collagen gels [15]. Therefore, gel contraction per se and the increased density of the matrix did not cause cessation of DNA synthesis. After short-term contraction of attached gels (1-4 d), fibroblasts were bipolar. On the other hand, after contraction of floating gels, fibroblasts were stellate [15]. The difference in cell shape can be understood in terms of asymmetry and the development of tension in the gels. In the case of attached collagen gels, the contractile force exerted by fibroblasts is distributed asymmetrically. Tension develops as indicated by the alignment of collagen fibrils parallel to the culture dish surface. The fibroblasts spread along the collagen fibrils and have a bipolar morphology. In the case of floating collagen gels, the contractile force exerted by fibroblasts is distributed symmetrically. The matrix remains relaxed as indicated by the nonorganized arrangement of collagen fibrils. The fibroblasts spread by extension of pseudopodia without adopting a particular polarity. In the present work, we observed a similar morphologic difference during the 4-week culture period; that is, bipolar cells in attached gels and stellate cells in floating gels. Therefore, growth of fibroblasts in attached collagen gels and regression of fibroblasts in floating collagen gels might depend on cell shape.

Growth of normal fibroblasts on planar surfaces [23] and growth of normal endothelial cells on extracellular matrix-coated surfaces [24] require cell spreading, although the mechanism by which cell spreading controls DNA synthesis is poorly understood. One aspect of control might be at the level of cellular oncogene expression [25]. Significantly, addition of platelet derived growth factor, fibroblast growth factor, or transforming growth factor-β did not restore normal levels of DNA synthesis to fibroblasts in floating collagen gels that had been contracted for 2 d [15].

The foregoing results suggest the novel idea that the regulation of fibroblast proliferation in granulation tissue by growth factors might depend on extracellular matrix organization and its influence on cell shape. In the early phases of wound healing, during granulation tissue formation, the wound bed would be asymmetric because of the fibronectin-rich wound interface [1,26]. Cells producing and contracting new extracellular matrix in this environment would be bipolar and responsive to growth factors. As production of new extracellular matrix replaces the wound interface, the tissue would regain its symmetrical organization. Then cells would become stellate and regress, returning to the state of fibroblasts in resting dermis [1,27]. The analogy is imperfect but provides a reasonable account for the cycle of growth activation and cessation during wound healing.

An unexpected feature of long-term cell cultures was the loss of starting collagen from both the attached gels and floating gels (70%-80%) during the 4-week culture period. Because the loss is cell dependent, it is likely to have occurred because of collagenase activity. Expression of collagenase activity in monolayer cell culture does not occur without modifying the cells or adding procollagenase activators [28,29]. Even in organ cultures, little collagenase activity was detectable in serum-containing medium [29]. The collagenase level normalized to cell number was much higher in floating gels than in attached gels, consistent with the increased expression of procollagenase message by fibroblasts in floating gels [30]. We have not attempted to isolate collagenase from the long-term cultures, and the enzyme might be bound to matrix rather than released in soluble form.

Another change in the long-term cultures was collagen processing. After 5 d, fibroblasts in attached collagen gels secreted procollagen into the medium. After 4 weeks, fibroblasts in attached collagen gels incorporated collagen α(I) chains into the matrix. Probably, collagen processing enzymes accumulated in the gels during this culture period [31,32]. That secreted proteins could diffuse out of the gels was shown by release of most fibronectin into the medium, both in 5-d cultures and in 4-week cultures. In fact, almost no fibronectin accumulated in the extracellular matrix of 4-week cultures. Perhaps there are a limited number of fibronectin binding sites on the collagen matrix, which were saturated early in the culture period.

There also were a variety of other differences between the proteins synthesized by fibroblasts after short-term and long-term culture in contracted gels. For instance, a major ~ 66 kDa component found in the medium of 5-d cultures was no longer synthesized in 4-week cultures. A pair of ~ 116 kDa components found in the medium of 4-week cultures was absent in 5-d cultures. Whether cells from 4-week cultures can revert to the earlier phenotype after release of the gels is an important question about the control of fibroblast differentiation by the extracellular matrix and one that we are now investigating.

Finally, it is important to note that all of the studies in this report were done with neonatal foreskin fibroblasts. Possibly, fibroblasts from adults or from other anatomical locations would behave differently in collagen gels.

Table I. Changes in Contracted Collagen Gels During Long-Term Culture*  

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<thead>
<tr>
<th>Feature</th>
<th>Attached gels</th>
<th>Floating gels</th>
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<tbody>
<tr>
<td></td>
<td>2 d</td>
<td>4 weeks</td>
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<tr>
<td>Gel contraction (% decrease in gel volume)</td>
<td>81</td>
<td>94</td>
</tr>
<tr>
<td>Cell number (109)</td>
<td>0.9</td>
<td>4.61</td>
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<tr>
<td>Cell shape</td>
<td>Bipolar</td>
<td>Bipolar</td>
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<td>Collagen degradation (% original collagen lost)</td>
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<td>83</td>
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<tr>
<td>Collagen synthesis pro α(I)</td>
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*See text for an explanation.

REFERENCES


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