Research article

Fibroblasts and monocyte macrophages contract and degrade three-dimensional collagen gels in extended co-culture

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Abstract

Background: Inflammatory cells are believed to play a prominent role during tissue repair and remodeling. Since repair processes develop and mature over extended time frames, the present study was designed to evaluate the effect of monocytes and fibroblasts in prolonged culture in three-dimensional collagen gels.
Methods: Blood monocytes from healthy donors and human fetal lung fibroblasts were cast into type I collagen gels and maintained in floating cultures for three weeks.
Results: Fibroblast-mediated gel contraction was initially inhibited by the presence of monocytes ($P < 0.01$). However, with extended co-culture, contraction of the collagen gels was greatly augmented ($P < 0.01$). In addition, with extended co-culture, degradation of collagen in the gels occurred. The addition of neutrophil elastase to the medium augmented both contraction and degradation ($P < 0.01$). Prostaglandin E_2 production was significantly increased by co-culture and its presence attenuated collagen degradation.
Conclusion: The current study, therefore, demonstrates that interaction between monocytes and fibroblasts can contract and degrade extracellular matrix in extended culture.

Keywords: Collagen degradation, IL-1, lung fibroblasts, monocytes, neutrophil elastase, PGE_2, TNF-α

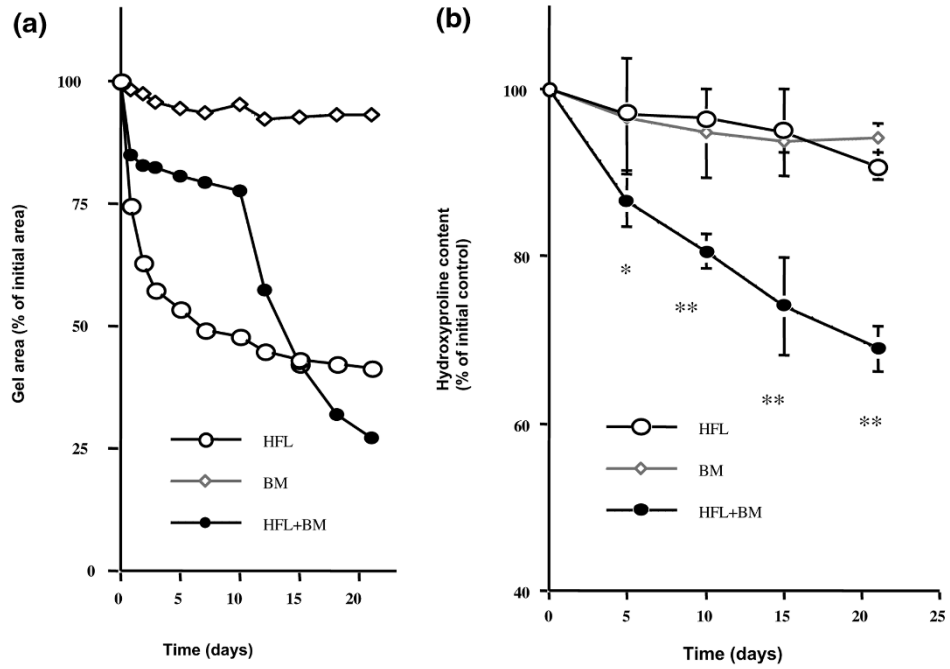
Introduction

Remodeling of extracellular structural elements is a prominent part of the response to injury. Imbalances in production and removal of extracellular matrices can lead to alterations in tissue structure that may compromise tissue function [1,2]. In the lung, inflammation is prominent in a variety of disease states associated with tissue remodeling, including the expansion and removal of extracellular matrix [1].

Three-dimensional (3D) matrix composed of reconstituted collagen fibers can create culture conditions more similar to *in vivo* tissues than routine dish cultures *in vitro* [3] and has been used to evaluate the remodeling process [4,5]. Co-cultures of monocytes with fibroblasts resulted in the inhibition of collagen gel contraction [4]. In contrast, co-cultures of fibroblasts with neutrophils or with neutrophil elastase (NE) resulted in augmentation of collagen gel contraction [5]. Over a three-day interval, neither elastase

3D = three-dimensional; EIA = enzyme immunoassay; ELISA = enzyme-linked immunosorbent assay; HFL = human fetal lung fibroblasts; IL-1β = interleukin-1β; NE = neutrophil elastase; PGE = prostaglandin E; SEM = standard error of the mean; TNF-α = tumor necrosis factor-α.

Figure 1



Effect of monocytes on fibroblast-mediated collagen gel contraction and degradation in long term co-culture. Fibroblasts (4×10^5/ml) or blood monocytes (4×10^5/ml) alone, or in combination, were cast into type I collagen gels and floated in serum-free DMEM. **(a)** The medium was changed every 5 days. Gel area was measured daily. Vertical axis: gel area expressed as percent of initial size. Horizontal axis: Time (days). **(b)** Replica gels were harvested on day 5, 10, 15 and 21, and hydroxyproline was quantified. Vertical axis: hydroxyproline content (percent of initial control). Horizontal axis: Time (days). BM, blood monocytes; HFL, human fetal lung fibroblasts. *$P < 0.05$ compared to baseline; **$P < 0.01$ compared to baseline.

nor the presence of monocytes was associated with degradation of the extracellular collagenous matrix [4]. The current study was designed to explore the effect of co-culture of monocytes and fibroblasts over extended time frames.

Materials and methods

See Supplementary material for further explanation of the methods mentioned below.

Cells and cultures

Human fetal lung fibroblasts (cell line HFL-1; American Type Culture Collection of Rockville, MD, USA) were passaged every week between cell passages 14 and 16. Blood monocytes were isolated from blood cells of healthy blood donors [6].

Preparation of collagen gels for 3D co-culture

Type I collagen was extracted from rat tail tendons and collagen gels were prepared as described previously [7].

Hydroxyproline assay

In order to determine the level of collagen degradation, the amount of hydroxyproline was measured by spectrophotometric determination [8,9].

Measurement of IL-1β, tumor necrosis factor-α and prostaglandin E_2

Concentrations of IL-1β, tumor necrosis factor (TNF)-α and PGE_2 were measured by ELISA or EIA assay.

Statistical evaluation

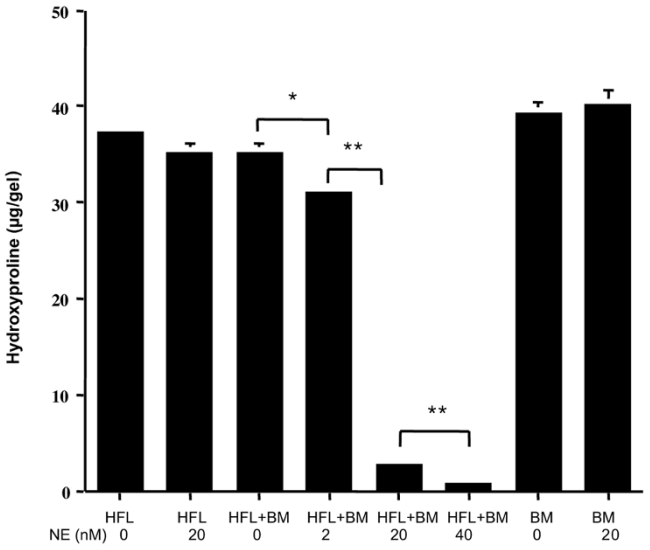
All results were confirmed by repeating experiments on separate occasions at least three times. For clarity, data shown in each figure were taken from single experiments and expressed as the means of three determinations ± SEM. Group data were evaluated by analysis of variance (ANOVA; StatView). Differences between two series of data that appeared statistically significant were analyzed by unpaired Student's *t*-test.

Results

Effect of monocytes on fibroblast-mediated collagen gel contraction and degradation

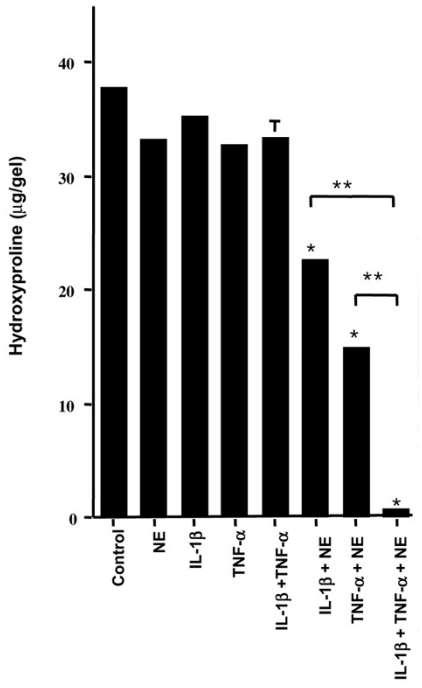
Blood monocytes, co-cultured with fibroblasts in 3D gels, attenuated the gel contraction for the first few days. On average, in three separate experiments, on days 3 and 7, fibroblasts alone contracted the gels to 53.1 ± 3.8% and 42.2 ± 4.1% of initial size. Monocytes reduced contraction to 79 ± 2.2% and 61 ± 11.1% of initial size, respectively (Fig. 1a, $P < 0.05$). At day 21, in contrast, the final size of

Figure 2



Effect of neutrophil elastase (NE) on collagen degradation in gels with co-cultured human fetal lung fibroblasts (HFL) and blood monocytes (BM). Gels were harvested on day 4 and collagen content was determined by measuring hydroxyproline. Vertical axis: Hydroxyproline content (µg/gel). Horizontal axis: culture conditions. *$P < 0.05$, **$P < 0.01$.

Figure 3



Effect of exogenous tumor necrosis factor (TNF)-α and interleukin (IL)-1β on fibroblast-mediated collagen degradation. Fibroblasts (4×10^5/ml) were cast into collagen gels and floated in serum-free DMEM to which TNF-α (10 ng/ml) and IL-1β (5 ng/ml) alone or in combination were added in the absence and presence of neutrophil elastase (NE; 20 nM). Hydroxyproline in the gels was determined on day 5. Vertical axis: hydroxyproline content (µg/gel). Horizontal axis: culture conditions. *$P < 0.01$, compared to control; **$P < 0.01$ between two groups.

the co-cultured gels was 21.0 ± 6.1% of initial area, and significantly smaller than that of the gels containing fibroblasts alone ($P < 0.05$). Monocytes alone did not contract the collagen gels.

Collagen content also was determined (Fig. 1b). Monocytes alone did not result in significant collagen degradation. Fibroblasts alone resulted in significant collagen degradation only at day 21 (10.1% of the initial collagen was degraded, $P < 0.05$). In contrast, co-culture of monocytes and fibroblasts resulted in significant degradation of collagen at all time points evaluated. By day 21, 30.9% of collagen in the gels was degraded in co-culture ($P < 0.01$).

Effect of neutrophil elastase on collagen degradation in co-cultured gels

The interaction between NE and monocytes on degradation of collagen in fibroblast-containing gels was evaluated. Over 4 days, NE had no effect on degradation of collagen in gels containing either monocytes alone or fibroblasts alone (Fig. 2). In contrast, NE added to co-cultures of fibroblasts and monocytes resulted in a concentration-dependent degradation of collagen.

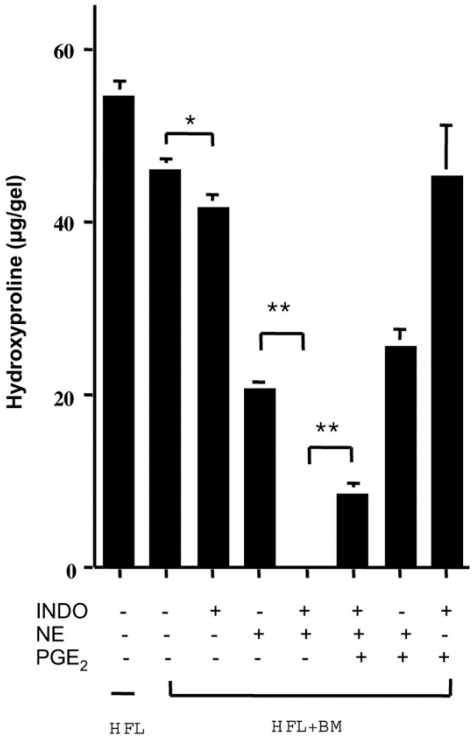
Role of IL-1β and tumor necrosis factor-α on degradation of collagen augmented by neutrophil elastase

Monocytes co-cultured with fibroblasts for 5 days resulted in the production of significant amounts of TNF-α (88.6 ± 4.9 ng/ml) and IL-1β (78.7 ± 5.5 ng/ml) while neither cytokine was detectable in cultures of monocytes or fibroblasts alone. To determine if these cytokines could contribute to the augmented degradation observed in co-culture, IL-1β and TNF-α were added to fibroblasts cultured in 3D collagen gels in the presence and absence of NE. In the absence of elastase, neither IL-1β nor TNF-α alone or in combination resulted in significant degradation of collagen. In the presence of elastase, IL-1β and TNF-α individually resulted in augmented degradation. IL-1β and TNF-α, together with elastase, resulted in nearly complete degradation of collagen (Fig. 3).

Role of PGE_2 in co-culture-mediated collagen degradation

Since both IL-1β and TNF-α can stimulate fibroblast 2 production, PGE_2 release was measured. Minimal production of PGE_2 (<1 ng/ml) was observed in cultures of monocytes (10^5/ml) or fibroblasts (10^5/ml) alone. In contrast, monocytes and fibroblasts in co-culture produced substantial amounts (10.2 ± 0.1 ng/ml; $P < 0.01$) of PGE_2. This production was maximal with the lowest number of

Figure 4



Effect of prostaglandin E_2 (PGE_2) on collagen degradation. On day 4, hydroxyproline in the gels was determined. Vertical axis: hydroxyproline content (µg/gel). Horizontal axis: culture conditions. BM, blood monocytes; HFL, human fetal lung fibroblasts; INDO, indomethacin; NE, neutrophil elastase. *$P < 0.05$; **$P < 0.01$.

monocytes tested, but demonstrated a clear dependency on the number of fibroblasts added (see Supplemental material). Indomethacin completely ($P < 0.01$) inhibited the PGE_2 release into the co-culture medium, while NE did not inhibit PGE_2 concentration (see Supplemental material). Consistent with a role for PGE_2 in modulating collagen degradation in co-cultures, indomethacin added alone slightly but significantly augmented collagen degradation (Fig. 4). Indomethacin added with NE resulted in complete collagen degradation, which was statistically greater than the 70% degradation observed with NE alone ($P < 0.01$). Conversely, exogenous PGE_2 significantly attenuated this augmented degradation. Exogenous PGE_2 also demonstrated a trend ($P = 0.07$) toward reducing the degradation that occurred with NE added to co-cultures in the absence of indomethacin. No significant effect occurred when PGE_2 was added with indomethacin in the absence of NE.

Discussion

Over a three-day period, co-cultures of monocytes with fibroblasts result in inhibition of fibroblast-mediated collagen gel contraction [4]. The current study extends these earlier observations in several important ways. In contrast to the initial inhibition of contraction, extended co-cultures resulted in augmented contraction. Furthermore, degradation of the collagen in the extracellular matrix occurred. The addition of NE to co-cultures resulted in concentration-dependent augmentation of collagen degradation. NE's effect must be indirectly related to regulation of other proteinases since degradation occurred in co-culture but not in cultures of fibroblasts or monocytes alone. Additionally, NE activity almost completely disappeared 12 hours after addition to cultures (data not shown).

Several studies have demonstrated an inverse relationship between the concentration of collagen contained in a gel and the degree of contraction [10,11]. The current study is consistent with these results and suggests that the augmented contraction observed in extended co-cultures may result from an alteration in the composition of the extracellular matrix, namely a reduction in the collagen content.

IL-1β and TNF-α are generally regarded as mediators responsible for initial events in an inflammatory process [12]. These same mediators, however, can clearly adjust the behavior of mesenchymal cells in a way that can alter tissue repair and remodeling. The current study provides additional evidence in support of these effects on tissue structure, and suggests that 'repair and remodeling' are processes initiated together with the earliest events in an inflammatory response. In this context, the induction of matrix metalloproteases (see accompanying article [13]), together with their activation by serine proteases present in an inflammatory milieu, may serve to eliminate the provisional matrix deposited early in an inflammatory event. Inflammatory mediators, therefore, may serve to inhibit the development of fibrosis. PGE_2 production was also stimulated by IL-1β and TNF-α, and PGE_2 appeared to downregulate collagen degradation. It seems likely, therefore, that a complex network of mediators can modulate inflammation-driven matrix remodeling.

Many chronic inflammatory diseases, including idiopathic pulmonary fibrosis, are associated with chronic inflammation. Inflammatory mediators have been regarded as potential drivers of the inflammatory response. Attempts to abrogate the fibrotic process using anti-inflammatory approaches, however, have had limited success. Also, recent research by Selman *et al.* [14] questions the role of inflammatory cells in idiopathic pulmonary fibrosis. The current study suggests that the role of inflammation and inflammatory mediators in a fibrotic process may have antifibrotic as well as profibrotic functions. Treatment of fibrosis, therefore, may require more complex strategies than simply inhibiting inflammation.

Monocytes are immature cells that undergo differentiation into macrophages [15]. To what degree monocyte differentiation occurred in the current study is unknown. It is

possible, therefore, that the shift from inhibition of fibroblast-mediated collagen gel contraction to augmented contraction is, in part, due to differentiation of monocytes within the culture. Evaluation of such a possibility will require characterization of monocyte phenotypes in co-cultures. Since fibroblasts are capable of releasing mediators that can alter leukocyte functions [16], the co-culture system offers a number of possibilities for complex interactions between the cells present. Fibroblasts cultured in 3D collagen gels did not change the contractile phenotype; for example, α-smooth muscle actin expression was not detectable (data not shown). However, in contrast to monolayer culture, 3D collagen gel culture modulated biosynthesis of fibronectin and cyclo-oxygenase-α at mRNA and protein levels. These results are under further investigation.

Conclusion

The current study demonstrates that monocytes and fibroblasts in extended co-culture can contract and degrade extracellular matrix. A linked study [13] takes these results further by demonstrating that the interactions between monocytes and fibroblasts lead to matrix metalloprotease production and that these interactions mediate tissue remodeling.

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Supplementary material

Materials and methods

Cells and cultures

Human fetal lung fibroblasts (cell line HFL-1), obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal-calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.25 μg/ml fungizone. The cells were cultured in 100 mm tissue culture dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA). The fibroblasts were passaged every week. Subconfluent fibroblasts were trypsinized (trypsin-EDTA; 0.05% trypsin, 0.53 mM EDTA-4 Na) and used for collagen gel culture. Fibroblasts used in these experiments were between cell passages 14 and 16. Blood monocytes were isolated from blood cells of healthy blood donors [6]. Cell suspensions were >96% monocytes by the criteria of cell morphology on Wright stained cytosmears. Monocytes were stored at 4°C and were used for co-culture within 4 hours after isolation.

Reagents

Human NE was purchased from ECP (Owensville, MO, USA). Human recombinant TNF-α and human recombinant IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). PGE_2 and indomethacin were purchased from Sigma (St. Louis, MO, USA). Tissue culture supplements and media were purchased from GIBCO (Life Technologies, Grand Island, NY, USA). Fetal calf serum was purchased from Biofluid (Rockville, MD, USA).

Tendons were excised from rat tails, and the tendon sheath and other connective tissue were removed carefully (rat tail tendon collagen). After repeated washing with Tris-buffered saline (TBS, 0.9% NaCl, 10 mM Tris, pH 7.5) and serial concentrations of ethanol (from 50% to 100%), type I collagen was extracted in 6 mM hydrochloric acid at 4°C for 24 hours. Protein concentration was determined

by weighing a lyophilized aliquot from each lot of collagen solution.

Preparation of collagen gels for 3D co-culture

For long-term co-culture, the medium was changed every 5 days. The areas of floating gels were measured using an image analyzer.

To demonstrate the effects of NE on collagen degradation, NE (2–40 nM) was added to the medium in which gels were floated. To investigate the effect of PGE_2 on collagen degradation, indomethacin (1 μM) or PGE_2 (0.1 μM) was added to the medium. To confirm that IL-1β and TNF-α could induce fibroblasts to degrade collagen, TNF-α and IL-1β were added to medium in which collagen gels containing fibroblasts alone were floated.

The appropriate amount of rat tail tendon collagen was mixed with distilled water, fourfold-concentrated DMEM, and cells were suspended so that the final mixture resulted in 0.75 mg/ml of collagen, with a physiological ionic strength and the desired cell concentration. Fibroblasts and monocytes were routinely added last. One-half ml of the mixture was cast into each well of 24-well tissue culture plates (Falcon, Franklin Lakes, NJ, USA). Gelation occurred at about 25 min at room temperature, after which the gels were released and transferred to 60 mm tissue culture dishes containing 5 ml of serum-free DMEM and cultured at 37°C, 5% CO_2. For long-term co-culture, the medium was changed every 5 days. The areas of floating gels were measured using an image analyzer (Optomax V, Optomax, Burlington, MA,USA).

Measurement of IL-1β, TNF-α and PGE_2

The concentration of IL-1β and TNF-α in media in which collagen gels were floated was measured by an enzyme-linked immunosorbant assay using commercially available materials (R&D System Inc, Minneapolis, MN, USA). The concentration of PGE_2 was assayed by EIA (PGE_2 EIA Kit, Cayman, Ann Arbor, MI, USA).

Hydroxyproline assay

The amount of hydroxyproline, which is directly proportional to the collagen content of the gels, was measured by spectrophotometric determination. Briefly, the media surrounding gels was completely removed, and the gels were transferred to glass tubes (KIMAX, Fisher Scientific, St. Louis, MO, USA) with 2 ml of 6 N HCl. Oxygen was removed by ventilating with N_2 for 30 s. The gels were then hydrolyzed at 110°C for 12 hours. The samples were dried with a vacuum centrifuge, and then dissolved in distilled H_2O. Hydroxyproline in the samples was reacted with oxidant (1.4% Chloramine T in acetate/citric acid buffer, Sigma, St. Louis, MO, USA) and Ehrlich's reagent (0.4% *p*-dimenthylamino-benzaldehyde, Sigma), in 60% perchloric acid, Fisher Chemical) at 65°C for 25 min, and the hydroxyproline content determined by measuring absorbance at 550 nm (Ultrospect 2000, Cambridge, England).

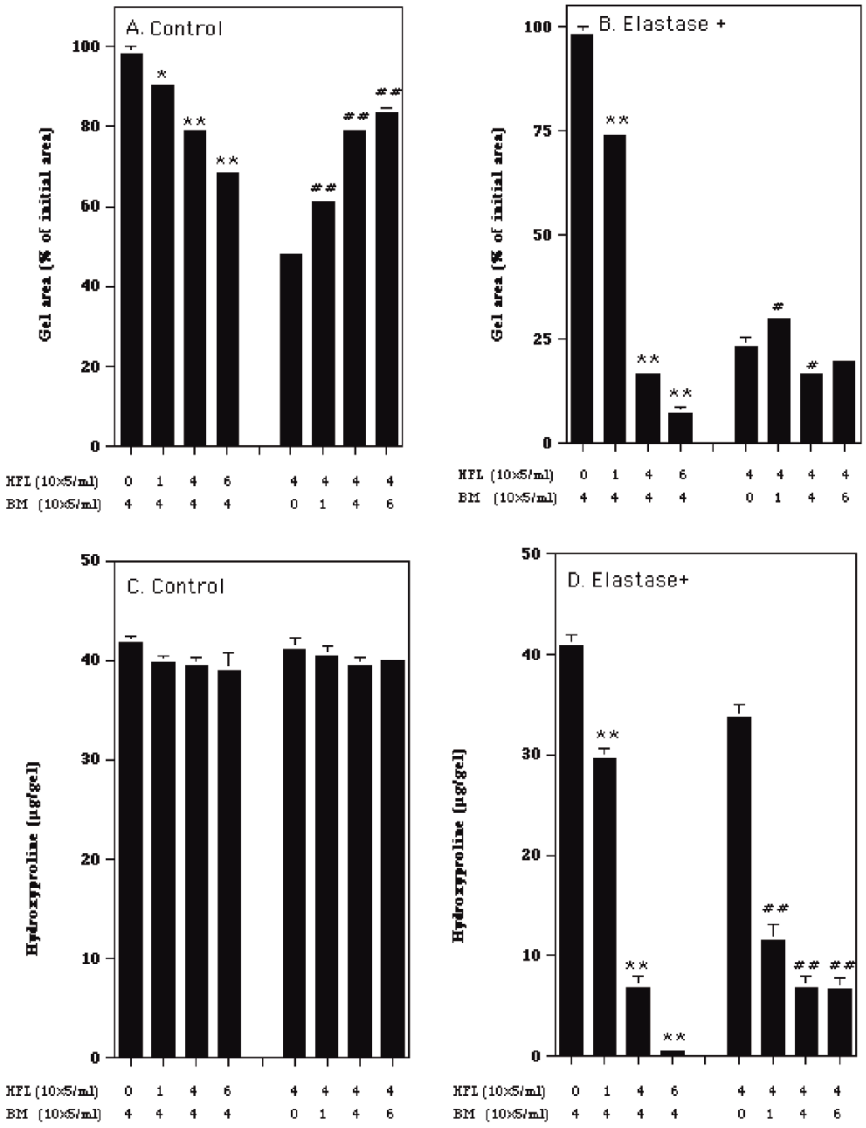
Results

Effect of cell number in co-cultured gels on gel contraction and collagen degradation

To determine the importance of relative cell number on gel contraction and collagen degradation in co-cultured gels, gels were prepared with either varying numbers of fibroblasts and a constant number of monocytes or, conversely, varying numbers of monocytes and a constant number of fibroblasts. In the absence of NE, increasing numbers of fibroblasts resulted in increasing contraction of the collagen gels (Supplementary Fig. 1a). In contrast, increasing numbers of monocytes resulted in inhibition of collagen gel contraction mediated by a constant number of fibroblasts (Supplementary Fig. 1a). In the presence of NE, collagen gel contraction continued to show a dependence on the number of fibroblasts present, although the amount of contraction was increased at each concentration of fibroblasts (Supplementary Fig. 1b). At 4 days, the monocyte number did not greatly affect collagen gel size in the presence of elastase (Supplementary Fig. 1b).

In the absence of elastase, minimal collagen degradation occurred at 4 days in gels containing any of the relative numbers of monocytes and fibroblasts tested (Supplementary Fig. 1c). In contrast, in the presence of NE, marked degradation of collagen occurred (Supplementary Fig. 1d). This was dependent on the number of both fibroblasts and monocytes added. Increasing numbers of fibroblasts were able to nearly completely degrade the gel collagen in the presence of a constant number of monocytes. In contrast, increasing numbers of monocytes appeared to result in a maximal degradation approaching 95% over the four-day culture in the presence of a constant concentration of fibroblasts.

Supplementary Figure 1



Effect of varying cell number on gel contraction and collagen degradation. Collagen gels were prepared with co cultures containing varying concentrations of either monocytes or fibroblasts. The gels were then floated in either serum-free DMEM (panels **a** and **c**) or in medium containing human neutrophil elastase (NE) (panels **b** and **d**). After 4 days, the gel area (panels a and b) and hydroxyproline content (panels c and d) were determined. Panels a and b: vertical axes = gel size on day 4 (% of initial area). Panels c and d: vertical axes = hydroxyproline content in the gels (µg/gel). Horizontal axes: fibroblast and monocyte densities (10^5/ml). The data shown are means ± SEM for triplicate determinations from a representative single experiment. BM, blood monocytes; HFL, human lung fibroblasts. $^*P < 0.05$, $^{**}P < 0.01$ compared to monocytes alone. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to fibroblasts alone.