

ALTERATIONS IN COLLAGEN PRODUCTION IN MIXED MONONUCLEAR LEUKOCYTE-FIBROBLAST CULTURES*

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The mechanism by which chronic inflammation leads to fibrosis and subsequent organ dysfunction is poorly understood. Disease states associated with fibrosing reactions such as progressive systemic sclerosis (PSS),¹ chronic graft vs. host disease, the fibrotic pulmonary disorders, and cirrhosis are often characterized by infiltration of mononuclear cells followed by either increased or altered collagen deposition (1). Several in vitro studies have indicated that mononuclear leukocytes (MNL) are capable of modulating the function of the cells of connective tissues, which suggests that cell-cell interactions are important in these processes (2-6).

There have been conflicting reports regarding the effects of supernatants from antigen- or mitogen-stimulated MNL cultures on fibroblast collagen production. It has been suggested (2) that the contradictory observations might be related to the different culture conditions used. Johnson and Ziff (3) observed stimulation of collagen synthesis by supernatants from cultures of phytohemagglutinin-stimulated MNL using human lung fibroblasts as the target cell. Supernatants from antigen-stimulated guinea pig peritoneal exudate cell cultures have been reported to stimulate both collagen production and fibroblast proliferation (4). In contrast, Jimenez et al. (2) have found that supernatants generated by cultures of human MNL stimulated with mitogens inhibited collagen production by dermal fibroblasts when incubated in the presence of serum. Using similar culture conditions to those of Jimenez and co-workers, Neilson et al. (5) reported that stimulation with renal tubular antigen of guinea pig mononuclear cells obtained from animals with interstitial nephritis generated soluble mediators that stimulated collagen production; supernatants from antigen-stimulated MNL from control animals inhibited collagen accumulation. More recently, fractionation of antigen-stimulated human MNL culture supernatants by molecular sieve chromatography has been reported to reveal mediators that stimulate collagen production as well as a substance that inhibits collagen accumulation (6). These findings suggest a multifaceted role for MNL in regulation of collagen production by the fibroblast.

These studies used MNL culture supernatants as the modulating agent and thus

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¹ *Abbreviations used in this paper:* FCS, fetal calf serum; MNL, mononuclear leukocytes; MEM, minimal essential media; NEM, *N*-ethylmaleimide; PMNL, polymorphonuclear leukocyte; PMSF, phenylmethanesulfonyl fluoride; PSS, progressive systemic sclerosis; TCA, trichloroacetic acid.

paid little attention to the possible effects of cell-cell interactions between the fibroblast and the MNL that would occur in the inflammatory milieu *in vivo*. Recently several investigators have assessed the effect of co-culture of mononuclear cells and connective tissue cells on alterations in connective tissue metabolism. Korn (7) has observed a short-term suppression of PHA-induced lymphocyte mitogenesis when lymphocytes are co-cultured with fibroblasts. Goldring et al. (8) reported an alteration in synovial cell responses to hormones when they were co-cultured with peripheral blood mononuclear cells. These studies suggest the possibility of cooperation between mesenchymal cells and the immune system in the control of connective tissue metabolism.

We have studied the effects on collagen production of co-culture of human fibroblasts with peripheral blood mononuclear cells. We elected to use the co-culture system to more closely reconstruct the *in vivo* milieu.

Materials and Methods

Mononuclear Cell Preparation. MNL were obtained from the peripheral blood of normal human volunteers by the Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO; Winthrop Laboratories, New York) technique (9).

Cell Culture. Dermal fibroblasts were obtained from explants of neonatal foreskins or punch biopsies from forearms of normal human volunteers by standard techniques. Human embryonic lung fibroblasts (HFL-1) were obtained from American Type Culture Collection (CCL 153, Rockville, MD). The cells were cultured in Eagle's minimal essential medium (MEM) with glutamine (2 mM) and Earle's salts supplemented by nonessential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml), ascorbic acid (50 µg/ml), and 9% fetal calf serum (FCS) (Microbiological Associates, Walkersville, MD) at 37°C in a humidified atmosphere of 5% CO₂/95% air.

All cultures used fibroblasts in the 4-14 passage. The fibroblasts were harvested using 0.25% trypsin and 1×10^5 cells/ml from a single pool were added to individual wells (2.1 cm²) of multiwell plates (Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, CA) in 0.5 ml media containing 9% FCS or 10-cm plates (Falcon Labware) in 16 ml of media containing serum. After the cells reached confluence (2-3 d), they were changed daily for 3 d into serum-free MEM supplemented only with antibiotics and ascorbic acid. Culture conditions minimized fibroblast proliferation during the experimental period.

Co-Culture for Collagen Production. Co-culture was initiated by the addition of the mononuclear cells in 0.5 ml of serum-free MEM to the confluent fibroblasts. After 24 h in the absence of serum, 2.5 µCi of [2,3-³H]proline (New England Nuclear, Boston, MA; sp act 20 Ci/mmol) was added to each well in a final volume of 0.5 ml of media and the cultures were continued for an additional 24 h. At the termination of the incubation, the supernatants were cleared of nonadherent cells by centrifugation and analyzed for collagen production.

Preparation of Co-Culture Supernatants. MNL (2×10^6 /ml) were co-cultured with confluent fibroblasts in a final volume of 8 ml. The supernatants from the cultures were harvested after 48 h of co-culture and nonadherent cells were cleared by centrifugation. Supernatants were tested for their ability to stimulate collagen production by adding an aliquot of the fraction to confluent fibroblasts in multiwell plates in a total volume of 0.5 ml of fresh serum-free MEM with 50 µg/ml of ascorbic acid and 2.5 µCi of [³H]proline. The duration of the incubation was 24 h. The supernatants from these secondary cultures were analyzed for collagen production.

Quantitation of Collagen Production. Collagen production was determined by a modification of the method of Peterkofsky and Diegelmann (10) using bacterial collagenase (Worthington Biochemical Corp., Freehold, NJ), which had been purified by molecular sieve chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ). After exhaustive dialysis to remove the unincorporated label, aliquots of the co-culture supernatants were incubated with and without purified bacterial collagenase (2 µg) for 90 min at 37°C in a reaction mixture containing 50 mM Tris-HCl/5 mM CaCl₂/2.5 mM *N*-ethylmaleimide (NEM), pH 7.6, in a final volume of 0.3 ml. After the addition of 0.025 ml of FCS as carrier protein, the reaction was terminated by the addition of an equal volume of cold 10% trichloroacetic acid (TCA)/

0.5% tannic acid, and the precipitated protein was cleared by centrifugation at 10,000 *g* for 5 min. An aliquot of the supernatant was counted in Instagel (Packard Instrument Co., Downers Grove, IL). The difference between counts per minute of ^3H released in the supernatant in the bacterial collagenase-digested samples and counts per minute of ^3H released in control samples represents bacterial collagenase-sensitive protein. The validity of the method was confirmed by comparison with [^3H]hydroxyproline determinations, after acid hydrolysis in 6 N HCl at 108°C for 24 h in an N_2 atmosphere, using a Technicon TM amino acid analyzer (Technicon Instruments Corp., Tarrytown, NY) equipped with a stream-splitting device.

Fibroblast number at the termination of the assay was determined by direct cell count after trypsinization using a hemocytometer. Each determination was done in duplicate. The results are expressed as collagen per well. Similar results were obtained if they were corrected for fibroblast cell number at the termination of the assay and results are expressed as [^3H]proline incorporation into bacterial collagenase-sensitive protein per 10^6 fibroblasts.

Non-Collagen Protein Synthesis. MNL-fibroblast co-cultures were prepared as described for co-culture for collagen production, but the cultures were labeled with 1.0 μCi of [^3H]tryptophan (New England Nuclear; sp act 5.96 Ci/mmol) in a final volume 0.5 ml serum-free media. Non-collagen protein synthesis was determined by precipitation of labeled culture supernatants with an equal volume of cold 10% TCA after the addition of carrier protein. The precipitate was collected on glass microfiber filters using a vacuum filtration manifold (Amicon Corp., Lexington, MA), washed twice with 5% TCA, air-dried, and then counted in Instagel. Determinations were done in duplicate.

Cell Layer Collagen. Confluent fibroblasts in serum-free media in 10-cm plates were co-cultured with 0.5×10^6 MNL/ml in 16 ml of serum-free MEM supplemented with ascorbic acid and antibiotics. After 24 h, 5.0 $\mu\text{Ci}/\text{ml}$ of [^3H]proline was added to each plate in a final volume of 16 ml of media, and the cultures were continued for an additional 24 h. At the end of the incubation, the media were removed and the cell layers were washed twice with phosphate-buffered saline containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM NEM, and 2.5 mM EDTA. The cell layer was removed from the culture plates by freeze-thawing three times in 0.1 M acetic acid. The cell layer was lyophilized and hydrolyzed in 6 N HCl at 108°C under an N_2 atmosphere. [^3H]Hydroxyproline content was determined as described above.

Molecular Sieve Chromatography of Co-Culture Supernatants. Supernatants from co-cultures were pooled and concentrated 60-fold at 4°C in an Amicon ultrafiltration cell with a UM2 Diaflo ultrafiltration membrane (Amicon Corp.). The concentrated supernatant was fractionated over a 1.5- \times 100-cm column of Sephadex G-200 at a flow rate of 5 ml/h. Proteins were eluted with 0.0075 M glycylglycine-NaOH/0.14 M NaCl, pH 7.2, at 4°C. The fractions were sterilized by filtration through a 0.45- μm Millipore filter (Millipore Corp., Bedford, MA). An aliquot of each fraction was added to fibroblast monolayers to determine its ability to stimulate collagen production. Incubations were identical to those with crude supernatants.

Determination of Collagen Type. Supernatants from [^3H]proline-labeled co-cultures and control cultures were harvested in the presence of 2.5 mM EDTA, 0.2 mM PMSF, and 10 mM NEM. The collagen fraction was precipitated using an equal volume of 50% ammonium sulfate after the addition of 0.5 mg/ml of cold carrier type I collagen. After exhaustive dialysis against 0.5 M acetic acid, the collagen was incubated with pepsin (100 $\mu\text{g}/\text{ml}$) for 18 h at 4°C to remove propeptides. The pepsin digestion was terminated by adjusting the pH to 8.0. At this stage, the solution contained a mixture of types I and III collagen. To determine the ratio of type I:III collagen, two different methods were used. The first method is that of interrupted electrophoresis as described by Sykes et al. (11). This method takes advantage of the fact that the $\alpha 1(\text{III})$ chains are linked together by disulfide bonds. Thus, $\alpha 1(\text{III})$ can be separated from $\alpha 1(\text{I})$ on sodium dodecyl sulfate (NaDoSO_4) gels by the delayed addition of a reducing agent. The second method makes use of the fact that native type III collagen is specifically cleaved into three-quarters and one-quarter fragments by human neutrophil elastase (12, 13). This enzyme has no effect on the helical region of native type I collagen. The three-quarter fragment of $\alpha 1(\text{III})$ can be easily separated from $\alpha 1(\text{I})$ by NaDoSO_4 gel electrophoresis. Specifically, after dialysis against 0.01 M Tris-HCl/0.15 M NaCl/0.02% sodium azide, pH 7.5, equal aliquots of the collagen preparations were incubated with either purified bacterial collagenase in the

presence of 5 mM NEM or purified polymorphonuclear leukocyte (PMNL) elastase in a reaction mixture containing 30 mM Tris-HCl/5 mM CaCl₂, pH 7.5, for 18 h at 27°C. The reaction was terminated by the addition of 5 mM EDTA and 1 mM PMSF, and the samples were lyophilized. The samples were dissolved in Laemmli sample buffer with 2-mercaptoethanol and were analyzed using 7.5% NaDoSO₄ polyacrylamide gel electrophoresis according to the method of Laemmli (14), using a slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). After fixation of the gels in 50% methanol/10% TCA, the gels were impregnated with Enhance (New England Nuclear) and fluorographed by standard techniques (15, 16), using prefogged Kodak X-AR-5 film (Eastman Kodak Co., Rochester, NY). Each track of the fluorographs was scanned using a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) equipped with a gel scanning attachment.

Interrupted electrophoresis gels were handled in an identical manner to the enzyme digestion gels. The percentage of type III collagen was determined by the following formula: percent type III collagen = $[\alpha 1(\text{III})]/[3/2 \alpha 1(\text{I}) + \alpha 1(\text{III})] \times 100$.

Results

Characterization of the Co-Culture System. After establishing that MNL-fibroblast co-culture stimulated the production of collagen, the effect of the duration of co-culture on collagen production was examined. MNL (10^6) were added to wells containing confluent fibroblasts and co-cultured in 0.5 ml of serum-free MEM for various time periods. Culture supernatants were analyzed for collagen production by a modification of the method of Peterkofsky and Diegelmann (10) as described in Materials and Methods. The results are seen in Fig. 1. Optimal enhancement of collagen production is noted at 48 h of co-culture after which there is a decline in stimulation of collagen accumulation though significant stimulation is still apparent. The reason(s) responsible for this decline in stimulation of collagen production at longer incubation periods are presently unclear but are under investigation.

To determine the effect of the quantity of MNL on the stimulation of collagen production, various numbers of MNL were added to a series of wells containing confluent fibroblasts, co-cultured for 48 h with a 24-h pulse period, and the supernatants analyzed for collagen production. Results are shown in Fig. 2. There is a direct correlation between increased collagen production and the number of MNL added. This stimulation of collagen accumulation is linear when MNL were added in

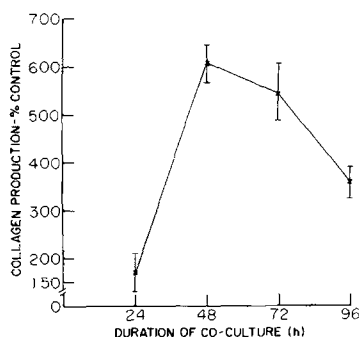


FIG. 1. Effect of duration of co-culture on collagen production. MNL (10^6) were added to confluent fibroblasts in 0.5 ml of serum-free MEM containing 50 $\mu\text{g}/\text{ml}$ of ascorbic acid and co-cultured for various time periods. Cultures were pulsed with 2.5 μCi of [^3H]proline 24 h before the termination of the co-culture. Supernatants were analyzed for collagen production. Determinations were done in quadruplicate and each point is expressed as mean \pm SD.

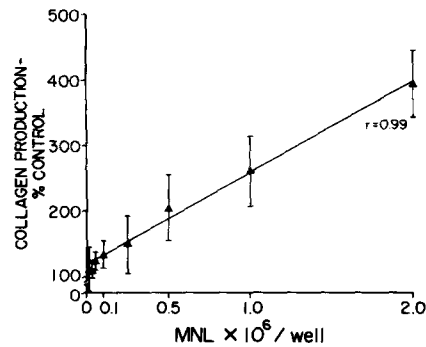


FIG. 2. Effect of MNL number on collagen production. MNL were added at increasing concentrations to confluent fibroblasts in 0.5 ml of serum-free MEM containing 50 $\mu\text{g}/\text{ml}$ of ascorbic acid and co-cultured for 48 h. [^3H]Proline was added for the final 24 h of co-culture. Culture supernatants were analyzed for collagen production. Determinations were done in quadruplicate and expressed as mean \pm SD.

the range of 10^4 – 2×10^6 cells/well. Heat-killed cells (15 min at 60°C) showed no stimulation of collagen accumulation (data not shown).

Because the mononuclear cells used in co-culture were routinely obtained from donors unrelated to the donor of fibroblasts, it was of interest to determine whether allogeneic differences between the fibroblast and MNL might be responsible for the stimulation of collagen production seen in this system. When confluent fibroblasts were co-cultured with MNL obtained from the donor of the fibroblast or MNL obtained from unrelated donors and the supernatants were analyzed for collagen production after a 48-h co-culture period, no significant differences in collagen production were noted between autologous and allogeneic cultures (data not shown). Thus, it appears that allogeneic differences between fibroblasts and MNL are not a major determinant in the generation of the stimulatory activity seen in this system.

Non-Collagen Protein Production. The increase in collagen production seen in the co-culture system could represent either a selective augmentation of collagen accumulation or a general increase in protein production by the fibroblast. To determine whether non-collagen protein production is stimulated by MNL-fibroblast co-culture, confluent fibroblasts were co-cultured with 10^6 MNL/well in the presence of 2.5 μCi of [^3H]proline or 1.0 μCi of [^3H]tryptophan, and the supernatants were analyzed for collagen or non-collagen protein production as described in Materials and Methods. As shown in Table I, non-collagen protein production was stimulated to a greater extent than collagen production. Non-collagen protein production was stimulated $\sim 150\%$ more than collagen accumulation. Though both MNL and fibroblasts contribute to non-collagen protein production in the co-culture samples, the data suggest that both collagen and non-collagen protein accumulation was enhanced.

Cell Layer Collagen. Because the increased collagen in the supernatants of the co-cultures could represent an increase in soluble collagen with a concomitant decrease in cell layer-associated collagen, the cell layer collagen from [^3H]proline-labeled control and co-cultures was quantitated. The lyophilized cell layer was analyzed for [^3H]hydroxyproline after acid hydrolysis as described in Materials and Methods. The collagen in the medium was quantitated as bacterial collagenase-sensitive protein. The results of these studies are shown in Table II. Bacterial collagenase-sensitive

TABLE I
*Stimulation of Collagen and Non-Collagen Protein Production by MNL-Fibroblast Co-Culture**

Cell line	MNL/well	Percent control protein production	
		Collagen	Non-collagen
F31C	0.5×10^6	198	285
F2D	0.5×10^6	179	277

* Confluent fibroblasts were cultured with 0.5×10^6 MNL/well for 48 h. During the final 24 h of incubation, cultures were incubated with either 2.5 μ Ci of [3 H]proline or 1.0 μ Ci of [3 H]tryptophan in a final volume of 0.5 ml of serum-free MEM. Supernatants were analyzed for collagen and non-collagen production as described in the methods. Data are expressed as the mean of duplicate determinations.

TABLE II
*Comparison of Stimulation of Collagen Production in Media and Cell Layer in MNL-Fibroblast Co-Culture**

	Percent control
Collagen, media	649
Collagen, cell layer	756

* Confluent fibroblasts were incubated with 0.5×10^6 MNL/ml for 48 h. During the final 24 h of incubation, the cultures were incubated with 2.5 μ Ci/ml of [3 H]proline. The culture supernatants were analyzed for collagen production ([3 H]proline incorporation into bacterial collagenase-sensitive protein). Cell layers were analyzed for [3 H]hydroxyproline after acid hydrolysis using an amino acid analyzer equipped with a stream-splitting device. Cell layer collagen represented <10% of the total collagen present in the cultures. Results are expressed as the mean value of duplicate determinations.

protein in the supernatant was stimulated 6.5-fold. Collagen accumulation in cell layer increased to a similar, though slightly greater, extent (7.5-fold). As cell layer collagen represents <10% of the total collagen present in fibroblast cultures, changes in the amount of cell layer collagen would not be expected to affect the media collagen significantly. However, these data clearly indicate that stimulation of collagen accumulation occurs both in the media and the cell layer of co-cultures; thus, the enhanced collagen accumulation in the media of co-cultures is not related to changes in solubility of the collagen.

Characterization of Collagen-stimulating Effect of Co-Culture Supernatants. Because it has been shown that antigen- and mitogen-stimulated MNL produce soluble mediators that enhance collagen production (3-6), supernatants from MNL-fibroblast co-culture were examined for their ability to stimulate collagen production. Supernatants were obtained by culturing 2×10^6 MNL/ml in 10-cm plates with confluent fibroblasts. Various dilutions of the co-culture supernatants were tested against different fibroblast monolayers in multiwell plates. The co-culture supernatants were incubated with confluent fibroblasts for 24 h in the presence of [3 H]proline. The newly labeled collagen in the supernatants of the secondary cultures was quantitated. The effect of dilutions of co-culture supernatants on collagen production is seen in Fig. 3. Optimal enhancement of collagen production was seen at a 1:10 dilution of the co-culture supernatant. Inhibition of collagen accumulation was noted only at 1:5 dilution.

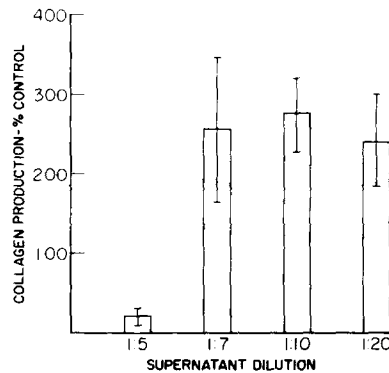


FIG. 3. Stimulation of collagen production by MNL-fibroblast culture supernatants. Co-culture supernatants were obtained by culturing 2×10^6 MNL/ml with confluent fibroblasts in 8 ml of serum-free MEM. Dilutions of co-culture supernatants were tested for ability to stimulate collagen production by incubating them with confluent fibroblasts in 0.5 ml of serum-free MEM in the presence of $50 \mu\text{g/ml}$ of ascorbic acid and $2.5 \mu\text{Ci}$ of [^3H]proline for 24 h. Determinations were done in quadruplicate and each point is expressed as mean \pm SD.

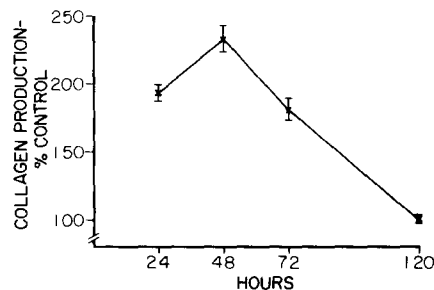


FIG. 4. Effect of duration of co-culture on formation of soluble factors that stimulate collagen production. Co-culture supernatants were generated by culturing 2×10^6 MNL/ml with confluent fibroblasts for various time periods. A 1:10 dilution of co-culture supernatant was tested for its ability to stimulate collagen production when incubated with confluent fibroblasts in 0.5 ml of serum-free MEM for 24 h in the presence of ascorbic acid ($50 \mu\text{g/ml}$) and [^3H]proline. Determinations were done in duplicate and each point is expressed as mean \pm SD.

Fibroblast-conditioned media did not stimulate collagen production (data not shown).

To determine the duration of co-culture necessary for the release of soluble mediators that stimulate collagen production into the co-culture supernatant, MNL ($2 \times 10^6/\text{ml}$) were co-cultured with confluent fibroblasts in 10-cm plates containing 8 ml serum-free MEM for various time periods. A 1:10 dilution of the co-culture supernatants was incubated with fibroblasts in multiwell plates to determine its ability to stimulate collagen production. The analysis of supernatants from secondary cultures for collagen production is seen in Fig. 4. Stimulation of collagen accumulation is maximal when supernatants from 48-h co-cultures are used. This corresponds to the optimal stimulation of collagen production in the direct MNL-fibroblast co-culture system even though the magnitude of stimulation is smaller.

Molecular Sieve Chromatography of Co-Culture Supernatants. To characterize further the soluble factors responsible for the stimulation of collagen production, supernatants from MNL-fibroblast co-cultures were fractionated on Sephadex G-200. Co-culture supernatants obtained by incubating 2×10^6 MNL/ml with confluent fibroblasts in

10-cm plates in 8 ml of serum-free media for 48 h were concentrated 60-fold by pressure dialysis and fractionated over a 1.5- × 100-cm column of Sephadex G-200 using 7.5 mM glycylglycine-NaOH/0.14 M NaCl, pH 7.2, as the eluting buffer. Fractions obtained were sterilized using a Millipore filter (0.45 μ m) before addition of 0.1 ml of the fraction to confluent fibroblasts in multiwell plates in 0.4 ml of serum-free MEM containing 50 μ g/ml of ascorbic acid and 2.5 μ Ci of [3 H]proline. After a 24-h incubation, the supernatants were analyzed for collagen production. A typical chromatogram is shown in Fig. 5. Major areas of activity are seen at \sim 160,000 and 80,000 mol wt. A lesser peak of activity is noted at a molecular weight of 25,000. These areas of activity could represent multiple molecular weight species of a single mediator in the form of aggregates or the effects of limited proteolytic digestion of larger molecular weight factor(s) by endogenous proteinases. An alternative possibility is that multiple soluble mediators capable of stimulating collagen production are generated in this system. Further work on the characterization of these soluble mediators is in progress.

Determination of Collagen Type. Because alterations in the ratio of type I:III collagen have been reported in several conditions including cirrhosis (17), idiopathic pulmonary fibrosis (18), and PSS (19), it was of interest to determine whether the stimulation of collagen accumulation seen in the co-culture system was associated with alterations in the relative amounts of types I and III collagen. Confluent fibroblasts were cultured with 0.5×10^6 MNL/ml in 10-cm plates for 48 h; 5.0 μ Ci/ml of [3 H]proline in a final volume of 16 ml of serum-free MEM supplemented with ascorbic acid (50 μ g/ml) was added during the final 24 h of culture. At the termination of the incubation, supernatants from control cultures and co-cultures were harvested in the presence of 0.2 mM PMSF, 10 mM NEM, and 2.5 mM EDTA, and the collagen was isolated as described in Materials and Methods. To separate α 1(I) from α 1(III), the collagen preparations were digested with human PMNL elastase for 18 h at 27°C. The reaction products were separated by NaDoSO₄ polyacrylamide gels, fluorographed, and quantitated by densitometry. Because PMNL elastase cleaves type III collagen but

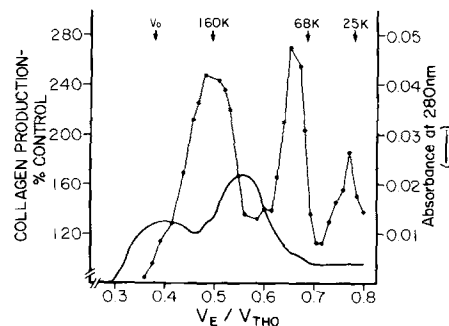


FIG. 5. Molecular sieve chromatography of co-culture supernatants. 120 ml of co-culture supernatant obtained by culturing 2×10^6 MNL/ml with confluent fibroblasts for 48 h were concentrated by ultrafiltration and fractionated over Sephadex G-200 (1.5- × 100-cm column) equilibrated in 0.0075 M glycylglycine-NaOH/0.14 M NaCl, pH 7.2. Aliquots (0.1 ml) of the column fractions were added to confluent fibroblasts to test their ability to stimulate collagen production. The mean of duplicate determinations are shown. Molecular weight markers were: V_0 , blue dextran; 160,000, IgG; 68,000, bovine serum albumin; 25,000, cytochrome *c*. Collagen production is expressed as percent control (buffer alone). Absorbance at 280 nm is indicated by the solid line.

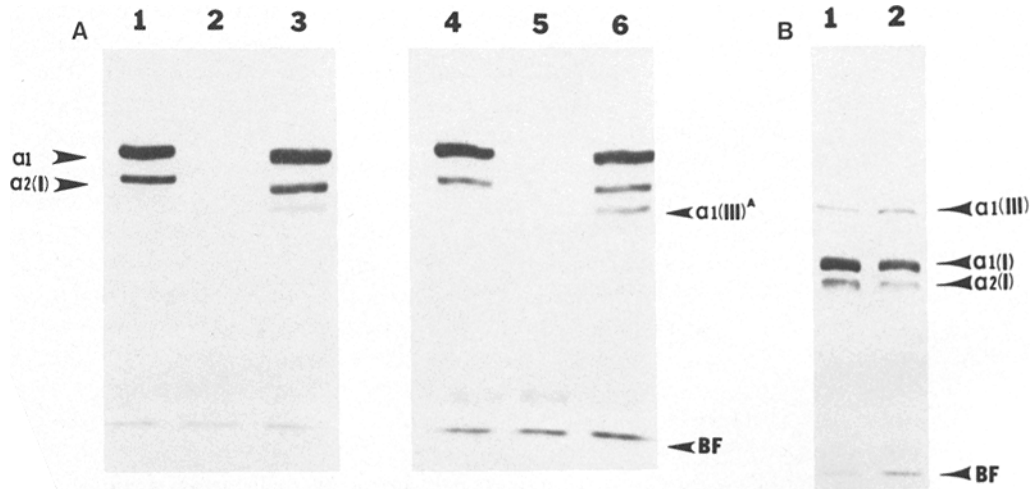


FIG. 6. PMNL elastase digestion and interrupted electrophoresis of collagen produced by fibroblast-MNL co-culture. (A) Pepsinized collagen from control and co-cultures were incubated with buffer (tracks 1 and 4), bacterial collagenase (tracks 2 and 5), or PMN elastase (tracks 3 and 6) for 18 h at 27°C. The reaction was terminated by addition of 5 mM EDTA and 1 mM PMSF before electrophoresis. Fluorographs of gels are shown. Tracks 1-3 represent control cultures and tracks 4-6 represent co-cultures. $\alpha 1$ in tracks 1 and 4 is a combination of both $\alpha 1(III)$ and $\alpha 1(I)$. (B) Interrupted electrophoresis of control cultures (track 1) and co-cultures (track 2).

not type I collagen, $\alpha 1(III)$ is converted to $\alpha 1(III)^A$, whereas type I collagen remains in the $\alpha 1$ position, which allows clear separation of these two $\alpha 1$ chains which would normally co-migrate on polyacrylamide gels. For comparison, $\alpha 1(I)$ and $\alpha 1(III)$ were also separated by the interrupted electrophoresis method of Sykes et al. (11). Representative fluorographs are seen in Fig. 6. Quantitation of the relative amounts of type I and III collagen present in culture supernatants revealed a consistent increase in the percentage of type III collagen in co-cultures as compared with controls (Table III). Both elastase digestion and interrupted electrophoresis gave similar results. Thus, the alteration in I:III ratio is not related to loss of type I collagen during the elastase digestion. The increase in the percent of type III collagen was seen in both dermal and lung fibroblast co-cultures (230 vs. 280% control, respectively). Similar results were obtained when β -aminopropionitrile was included in the cultures.

Discussion

The present study describes a system that uses the co-culture of human dermal fibroblasts with peripheral blood mononuclear cells to study the cellular interactions which promote increased collagen accumulation. It is apparent from the data that co-culture of MNL and fibroblasts results in a stimulation of collagen production and the magnitude of enhancement correlates directly with the number of MNL added. The stimulation of collagen accumulation is most evident when a 48-h co-culture period is used. The increased collagen in the media is paralleled by an enhanced deposition of collagen in the cell layer; thus, the observed stimulation of collagen production is not related to alterations in the solubility of the collagen that could cause enhanced release of collagen into the media rather than deposition in the cell layer matrix.

TABLE III
*Collagen Type Produced in MNL-Fibroblast Co-Cultures**

	Percent type III
Human dermal fibroblasts	
Elastase digestion	
Control	6.4
Co-culture	14.8
Human lung fibroblasts	
Elastase digestion	
Control	5.3
Co-culture	14.7
Interrupted electrophoresis	
Control	5.6
Co-culture	16.4

* Pepsinized collagen isolated from the culture medium by ammonium sulfate precipitation was incubated with either bacterial collagenase in a reaction mixture containing 30 mM Tris-HCl/5 mM CaCl₂/2.5 mM NEM, pH 7.5, or PMNL elastase in a reaction mixture containing 30 mM Tris-HCl/5 mM CaCl₂, pH 7.5, in a final volume of 0.3 ml for 18 h at 27°C. Reaction products were separated by electrophoresis on NaDoSO₄ polyacrylamide gels. PMN elastase selectively cleaves type III collagen yielding α1(III)^A, which can be easily separated from α1(I) on NaDoSO₄ polyacrylamide gels. After electrophoresis, the gels were fluorographed and then quantitated by densitometry. Interrupted electrophoresis was performed by the method of Sykes et al. (11). In this method, the delayed addition of a reducing agent allows the separation of α1(III) from α1(I), which would normally co-migrate. The samples used for interrupted electrophoresis were handled in parallel with the elastase digested samples. Percentage of type III collagen is expressed as $[\alpha 1(\text{III})]/[3/2 \alpha 1(\text{I}) + \alpha 1(\text{III})] \times 100$.

Although non-collagen protein production is also increased in co-cultures, it is difficult to determine the relative contribution of the fibroblast and the MNL in this enhancement. Although the MNL when incubated alone incorporate little tryptophan, the amount of protein they produce after stimulation in the co-culture system is not known. It is conceivable, however, that the co-culture system could cause a general activation of the fibroblast synthetic mechanisms. In studies not reported here, we have observed that glycosaminoglycan accumulation is also stimulated by fibroblast-MNL co-culture. Wahl et al. (4) noted an increase in both collagen and non-collagen protein production when guinea pig fibroblasts were stimulated with supernatants from peritoneal exudate cell cultures.

Soluble mediators capable of stimulating collagen production can be detected in co-culture supernatants, which is consistent with the findings of others (3, 4, 6). The time course of release of soluble mediators parallels the activity of the co-culture system; however, the magnitude of stimulation is less than that seen with the direct co-culture system. When co-culture supernatants are fractionated over Sephadex G-200, multiple areas of stimulatory activity are seen. The peak of activity seen at 160,000 mol wt may be similar to the collagen-stimulating protein described by Postlethwaite et al. (6) found in culture supernatants of antigen-stimulated human lymphocytes.

Several differences should be noted between the actual co-culture system and the

use of co-culture supernatants to induce increased collagen production. Although enhancement of collagen production is linear with the number of MNL added, stimulation of collagen production by culture supernatants appears to have a distinct optimum. In contrast to systems using antigen-stimulated MNL culture supernatants to stimulate collagen production (6), co-culture supernatants appear to inhibit collagen production only at very high concentrations. These findings suggest that cell-cell interactions may be important in the generation of soluble factors that influence collagen production. The difference in the magnitude of stimulation of collagen production between the co-culture system and the soluble mediators generated by co-culture implies that cell-cell interaction is important in this phenomenon. Whether cell-cell interactions serve to potentiate the response of the fibroblast to soluble factors or are capable of directing increased collagen production remains to be elucidated.

Quantitation of the relative amounts of types I and III collagen produced by fibroblasts when stimulated by co-culture with MNL revealed a relative increase in the type III collagen present (or a decrease in the I:III ratio). A decrease in type I:III ratio is most commonly associated with normal wound healing (20) though it has also been reported to occur in certain inflammatory states (21, 22). In contrast, an increased type I:III ratio has been found reported in certain fibrotic conditions (17, 18). It has been suggested, however, that type III collagen may be increased early in many types of inflammation, although this is not detected later in disease processes when pathologic specimens are available for analysis (23).

The decrease in the type I:III ratio seen in the co-culture system could be attributed to several mechanisms. Endogenous proteinases in the co-culture system could degrade type I collagen more readily than type III collagen. Although latent collagenolytic activity can be detected in co-culture supernatants, active collagenolytic activity has not been seen (data not shown). This does not exclude the possibility of cell surface or cell-associated proteinases that could degrade type I collagen more avidly than type III. However, because typical interstitial collagenases degrade type III collagen preferentially, this seems unlikely (24). Neutrophil collagenase has been reported to have a greater activity against type I collagen (25), but since neutrophil contamination in the MNL preparation was <1%, it is doubtful that significant neutrophil collagenolytic activity was present.

Another explanation for the observed alteration in the type I:III ratio could be that the fibroblast itself may control the relative proportion of types I and III collagen produced. The observation that cell-associated dialyzable hydroxyproline is altered by various stimuli has prompted the suggestion that newly synthesized collagen may be destroyed in the cell before secretion (26). Increased intracellular concentrations of cyclic AMP have been reported to cause a decrease in type I collagen production by fibroblasts accompanied by a decreased type I:III ratio (27). Type I procollagen mRNA in the presence of increased intracellular levels of cAMP showed no alteration; however, dialyzable hydroxyproline increased, which suggests that the decrease in collagen production seen was secondary to intracellular wastage of type I collagen (28). Although further studies are needed, it is conceivable that the fibroblast responds to MNL by modulating not only the amount of collagen produced but also the types of collagen secreted. Because the alteration of type I:III ratio may be associated with changes in tissue compliance and subsequent organ dysfunction, it appears important to determine the relative amounts of type I and III collagens produced by experimental

systems.

Summary

The cell-cell interactions between fibroblasts and mononuclear leukocytes (MNL) which promote alterations in collagen accumulation were examined using a system of co-culture of human fibroblasts and peripheral blood MNL. The stimulation of collagen production was optimal after 48 h of co-culture and the increase in collagen correlated directly with the number of MNL added. The enhancement of collagen production was seen in both autologous and allogeneic co-cultures. Stimulation of non-collagenous protein was also noted. Co-culture supernatants contained soluble substances that were capable of stimulating collagen production, although they stimulated collagen production to a lesser degree than direct co-culture. Fractionation of these supernatants on Sephadex G-200 revealed a predominant area of stimulatory activity at 160,000 mol wt. Lesser areas of activity were noted at molecular weights of 80,000 and 25,000. Determination of the types of collagen produced by fibroblasts during co-culture with MNL showed that the ratio of type I:III collagen was decreased. These alterations in both the quantitative and qualitative accumulation of collagen mimic the changes often seen in wound healing and early inflammation suggesting that cellular interactions between fibroblasts and MNL may be important in the modulation of collagen production in normal and pathologic states.

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References

1. Sporn, M. B., and E. D. Harris. 1981. Proliferative diseases. *Am. J. Med.* **70**:1231.
2. Jiminez, S. A., W. McArthur, and J. Rosenbloom. 1979. Inhibition of collagen synthesis by mononuclear cell supernatants. *J. Exp. Med.* **150**:1421.
3. Johnson, R. L., and M. Ziff. 1976. Lymphokine stimulation of collagen accumulation. *J. Clin. Invest.* **58**:240.
4. Wahl, S. M., L. M. Wahl, and J. B. McCarthy. 1978. Lymphocyte-mediated activation of fibroblast proliferation and collagen production. *J. Immunol.* **121**:942.
5. Neilson, E. G., S. A. Jiminez, and S. M. Phillips. 1980. Cell-mediated immunity in interstitial nephritis. II. T lymphocyte-mediated fibroblast proliferation and collagen synthesis; an immune mechanism for renal fibrogenesis. *J. Immunol.* **125**:1708.
6. Postlethwaite, A. E., G. N. Smith, C. L. Mainardi, J. M. Seyer, and A. H. Kang. 1981. Characterization of a human lymphokine that stimulates fibroblasts to produce collagen. *Arthritis Rheum.* **24**:S61.
7. Korn, J. H. 1981. Modulation of lymphocyte mitogen responses by co-cultured fibroblasts. *Cell. Immunol.* **63**:374.
8. Goldring, S. R., J. M. Dayer, and S. M. Krane. 1981. Synovial cell hormone responses modulated by cell-cell interactions. *Arthritis Rheum.* **24**:S106.
9. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood: isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Invest.* **21**(Suppl. 97):77.
10. Peterkofsky, B., and R. Diegelmann. 1971. Use of mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry.*

- 10:988.
11. Sykes, B., B. Puddle, M. Francis, and R. Smith. 1976. The estimation of two collagens from human dermis by interrupted gel electrophoresis. *Biochem. Biophys. Res. Commun.* **72**:1472.
 12. Mainardi, C. L., D. L. Hasty, J. M. Seyer, and A. H. Kang. 1980. Specific cleavage of human type III collagen by human polymorphonuclear leukocyte elastase. *J. Biol. Chem.* **255**:12006.
 13. Gadek, J. E., G. A. Fells, D. G. Wright, and R. G. Crystal. 1980. Human neutrophil elastase functions as a type III collagen "collagenase." *Biochem. Biophys. Res. Commun.* **95**:1815.
 14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond.)*. **227**:680.
 15. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83.
 16. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ³H- and ¹⁴C- in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335.
 17. Seyer, J. M., E. T. Hutcheson, and A. H. Kang. 1977. Collagen polymorphism in normal and cirrhotic human liver. *J. Clin. Invest.* **59**:241.
 18. Seyer, J. M., E. T. Hutcheson, and A. H. Kang. 1976. Collagen polymorphism in idiopathic chronic pulmonary fibrosis. *J. Clin. Invest.* **57**:1498.
 19. Gay, R. E., R. B. Buckingham, R. K. Prince, S. Gay, G. P. Rodnan, and E. J. Miller. 1980. Collagen types synthesized in dermal fibroblast cultures from patients with early progressive systemic sclerosis. *Arthritis Rheum.* **23**:190.
 20. Barnes, M. J., L. F. Morton, R. C. Bennett, A. J. Bailey, and T. J. Sims. 1976. Presence of type III collagen in guinea pig dermal scar. *Biochem. J.* **157**:263.
 21. Weiss, J. B., C. A. Shuttleworth, R. Brown, K. Sedowfia, A. Baildan, and A. Hunter. 1975. Occurrence of type III collagen in inflamed synovial membranes: a comparison between non-rheumatoid, rheumatoid and normal synovial collagens. *Biochem. Biophys. Res. Commun.* **65**:907.
 22. Bailey, A. J., T. J. Sims, M. Lelouis, and S. Bazim. 1975. Collagen polymorphism in experimental granulation tissue. *Biochem. Biophys. Res. Commun.* **66**:1160.
 23. Bornstein, P., and H. Sage. 1980. Structurally distinct collagen types. *Annu. Rev. Biochem.* **49**:957.
 24. Welgus, H. B., J. J. Jeffrey, and A. Z. Eisen. 1980. The collagen substrate specificity of human skin fibroblast collagenase. *J. Biol. Chem.* **256**:9511.
 25. Horwitz, A. L., A. J. Hance, and R. G. Crystal. 1977. Granulocyte collagenase: selective degradation of type I relative to type III collagen. *Proc. Natl. Acad. Sci. U. S. A.* **74**:897.
 26. Bienkowski, R. S., B. J. Baum, and R. G. Crystal. 1978. Fibroblasts degrade newly synthesized collagen within the cell before secretion. *Nature (Lond.)*. **276**:413.
 27. Rennard, S., L. Saltzman, J. Moss, G. Fells, J. Gadek, B. Horn, G. Hunninghake, and R. Crystal. 1981. Modulation of fibroblast production of collagen types I and III: effects of PGE₁ and isoproterenol. *Fed. Proc.* **40**:1813.
 28. Berg, B. A., P. Tolstoshev, J. Moss, and R. G. Crystal. 1981. Suppression of collagen production by acute exposure of fibroblasts to β -agonists is not accompanied by a decrease in collagen mRNA levels or activity. *Fed. Proc.* **40**:1813.