Effect of Soluble Products from Lectin-stimulated Lymphocytes on the Growth, Adhesiveness, and Glycosaminoglycan Synthesis of Cultured Synovial Fibroblastic Cells

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ABSTRACT Human blood mononuclear cells exposed to concanavalin A or phytohemagglutinin secrete a soluble factor that arrests the growth of human synovial fibroblastic cells in culture. Once the growth-inhibitory effect is initiated it cannot be reversed by washing the fibroblastic cells, by refeeding with non-conditioned fresh serum-containing medium, by trypsinization, EDTA treatment, or a combination of these procedures. Media from nonstimulated mononuclear cells, fibroblastic cells, or the lectins themselves do not contain similar inhibitory activity that can be detected by the present culture systems. This lectin-dependent, growth-inhibitory activity does not have a cytotoxic effect on the fibroblasts but increases their adhesiveness to plastic or glass surfaces, and the cells tend to assume a less fibroblastic morphology. The growth-inhibitory activity is stable in the cold and is nondialyzable or ultrafilterable, but the activity is rapidly lost at temperature between 60° and 70°C and at pH 2.0. The growth-arrested cells secrete more glycosaminoglycan per cell in the medium and synthesize more cell surface glycosaminoglycan than the controls. However, the increased glycosaminoglycan synthesis cannot be explained as being entirely secondary to a cell density effect as it is also observed when adjustments are made for the differences in growth rates.

INTRODUCTION

The increased proliferation of synovial lining cells occurring in association with the appearance and persistence of lymphocytes in the subsynovium are well recognized histological features of early rheumatoid arthritis (1, 2). We had shown (3) that early subcultures of synovial fibroblastic cells derived from synovial explants from inflammatory arthropathies have relatively high rates of growth and achieve higher saturation densities than synovial fibroblastic cells from noninflammatory arthropathies. However, the culture techniques used tend to select fibroblastic populations that proliferate most rapidly on plastic surfaces, and this proliferation is in turn partially dependent on the rate of diffusion of nutrient molecules and metabolites (4), on pH (5), and on the serum concentration and feeding protocols that are used (3). It is likely, therefore, that synovial fibroblastic cell populations whose proliferative growth within rheumatoid synovium has been arrested or diminished would not be "seen" in experiments that rely on fibroblastic outgrowths from synovial tissue explants. Thus, effects of products from stimulated lymphocytes in rheumatoid tissues (6) that could suppress the growth and alter the metabolism of synovial fibroblastic populations might not be detected by the synovial explant technique.

Biological activities that stimulate fibroblastic growth and hyaluronic acid synthesis have been described and characterized to varying degrees (7–9). A small molecular weight peptide from platelets stimulates the growth of human synovial fibroblasts (10), and a similar connective tissue-activating peptide from lymphocytes (11) stimulates glycosaminoglycan secretion of synovial fibroblastic cells, but its presence in human lymphocyte cultures does not appear to be mediated by plant lectin mitogens.

A mitogen-dependent factor from stimulated lymphocytes that affects synovial fibroblastic growth has

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not been described. We pursued this possibility as part of a systematic investigation of the interactions between lymphocytes stimulated by lectins and synovial fibroblasts. We now report the presence of a factor that suppresses synovial fibroblastic cell growth and alters the metabolic and adhesive properties of synovial fibroblastic cells.

METHODS

Establishment of synovial fibroblastic cells. Synovial tissue (0.5–1 g) obtained at arthrotomy was rinsed in serum-free (Connaught Medical Research Laboratories) 1969 medium (Connaught Laboratories, Toronto, Canada), then minced as finely as possible and suspended in 10 ml of a solution containing 10 mg/ml collagenase (From Cl. histolyticum, "CLS; Type IV," Worthington Biochemical Corp., Freehold, N. J.). The tissue digest was incubated for ~4 h at 37°C with periodic mixing using a pasteur pipette. After this incubation, 10 ml of a solution consisting of 0.05% trypsin (Sigma Chemical Co., St. Louis, Mo.) and 0.1% EDTA Na_2 (Fisher Scientific Co., Pittsburgh, Pa.) made up in 1969 serum-free medium was added. The tissue digest was again mixed and incubated 45 min further. The digest was then centrifuged for 5 min with a clinical desk centrifuge at ~2,000 rpm and the cell pellet was suspended and washed again in serum-free 1969 medium followed by resuspension in 1969 medium containing 20% fetal calf serum. The suspension was then seeded into 35-mm dishes (1.5–2.0 ml/dish). The cells were subcultured by described methods (3) and were used for experiments between the third and seventh subculture. After a trypsinization procedure using a 0.25% trypsin solution (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), (3) the cells were counted with an electronic counter (model ZF, Coulter Electronics, Inc., Hialeah, Fla.) set at TL 50, A-2, current 4.

Synovial fibroblast growth experiments. Initial growth experiments were carried out in 35-mm dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). However, for the growth experiments described in detail under Results, the growth curves were carried out in 16-mm Linbro wells (Linbro Chemical Co., Hamden, Conn.) unless otherwise indicated. The following protocol was adopted, unless otherwise indicated: the fibroblasts were seeded at a plating density of ~5,000 cells per well in 1 ml of 1969 medium suplemented with 20% fetal calf serum (Grand Island Biological Co.). After the cells were allowed to attach (24 h) they were refed with 0.8 ml of 1969 medium supplemented with 20% fetal calf serum and 0.2 ml of the medium or fraction to be tested or 0.2 ml of the appropriate control medium. The time point of the addition of the medium containing the test of control fractions is designated as the beginning of day 1. The fibroblasts were enumerated at various time points thereafter as described above.

Preparation of conditioned media. 10-ml samples of normal human venous blood was collected in sterile, heparinized tubes. 4 ml of blood was layered on top of 3 ml Ficoll-Paque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), and the tubes were centrifuged at 400 *g* for 30 min at room temperature. The lymphocyte-rich layer was removed and washed twice with a balanced salt solution consisting of Dulbecco's phosphate-buffered saline (PBS) (12) supplemented with glucose 1 g/liter; $CaCl_2$, 0.10 g/liter, and $MgCl_2 \cdot 6H_2O$, 0.1 g/liter. The lymphocyte-rich pellet was collected each time using a clinical desk centrifuge at setting number 6 and centrifuging for 10 min. The cells were then suspended in 10 ml of 1969 medium containing 20% decomplemented fetal calf serum and enumerated on the electronic counter (set at TL 5, A-2, current 2). The cells were then diluted in the same medium to give a final concentration of ~10^6 cells/ml. 2 ml of the above lymphocyte suspension was plated into each 35-mm plastic tissue culture dish. 0.5 ml of serum-free 1969 medium with or without mitogen was added to the dishes and the incubation was allowed to proceed for 72 h at 37°C in a CO_2 incubator. At the end of the incubation period the cells were separated from the media supernate by spinning 10 min at speed 6 in the clinical centrifuge. The supernate (2–2.5 ml) was filtered through 20-μm pore acrodiscs (Gelman Instrument Co., Ann Arbor, Mich.). The plant mitogens used were as follows: phytohemagglutinin—M (PHA),[1] extracted from the kidney bean—grade B, and concanavalin A (Con A) extracted from the jack bean—grade A, were purchased from Calbiochem-Behring Corp., San Diego, Calif. The amounts of mitogens that were added to the lymphocyte cultures in 0.5 ml of serum-free medium were 62.5 μg of Con A and 125 μg of PHA.

Media that were "conditioned" with the lymphocyte-rich preparation in the absence of mitogens are designated as "non-stimulated lymphocyte medium," and those where mitogens were used are designated as "PHA-stimulated lymphocyte medium" and "Con-A-stimulated lymphocyte medium" for the PHA and Con A mitogens, respectively. Media were also conditioned with synovial fibroblastic cells in the following way: synovial fibroblastic cells were allowed to grow to confluence in 35-mm plastic dishes and were then refed with 2.0 ml of serum-containing medium that was collected after 72 h and designated as conditioned fibroblast medium. Controls of serum-free medium or the mitogens in 0.5 ml of serum-free medium and unconditioned 20% serum-containing medium were also used.

Separation of adherent cells in the lymphocyte-rich preparation. In a typical experiment 2.6×10^7 cells were obtained by the Ficoll-Paque procedure as described above. After being washed, the cell pellet was resuspended in 10 ml of 1969 serum-containing medium as described above, and the cells were further diluted in the same medium and plated in 35-mm plastic dishes at a final cell density of 2.2×10^6 cells per dish in 2 ml of medium. After 24 h, nonadherent cells were recovered from half of the dishes by gently rinsing the dishes with serum-free medium. The lymphocytes were then centrifuged, the pellet resuspended, and the cells counted and adjusted to the same final concentration (2.2×10^6 cells per dish) as that in the remaining dishes that included adherent cells. Recovery of nonadherent cells was about 86% of the initial lymphocyte-rich preparation by the Ficoll-Paque procedure. Con A, at the concentration indicated above, was added in 0.5 ml of serum-free medium to some of the dishes of both the nonadherent cells and the initial lymphocyte-rich groups. Serum-free medium alone (0.5 ml) was added to other dishes from each group that served as the non-stimulated controls for the lymphocyte-rich preparation and for this preparation depleted of adherent cells.

Glycosaminoglycan secretion studies. The quantitation of glycosaminoglycan production by the cultured synovial cells was carried out as described (13–16). Briefly, synovial fibroblastic cells obtained from patients with rheumatoid arthritis were seeded in 35-mm dishes or 16-mm wells at known initial plating densities. For some experiments, on

[1] *Abbreviations used in this paper*: Con A, concanavalin A; lymphocytes, lymphocyte-rich preparation from human peripheral blood; PBS, phosphate-buffered saline; PHA, phytohemagglutinin-M.

day 1, the medium was removed, and the cells were refed with either 2 ml (for 35-mm dishes) or 1 ml (for 16-mm wells) of fresh medium containing 1 μCi/ml of D-(1,6)[^{3}H]glucosamine, sp act 21 Ci/mmol (New England Nuclear, Boston, Mass.), unless otherwise indicated. In some experiments where incorporation of radioactivity into the glycosaminoglycans was only measured, radioactive glucosamine was allowed to be in contact with the cells during a "prelabeling" period of at least 2 d to allow equilibration of glycosaminoglycan precursor pools with the label. The media were then replaced with fresh serum-containing media with the same amount of radioactive glucosamine and supplemented with the various conditioned media in a proportion of conditioned medium/fresh serum-containing medium, 1:5. In experiments where specific activities and net rates of glycosaminoglycan synthesis were determined, no prelabeling period was used. At the time intervals indicated under Results, the media were removed and stored at −10°C for subsequent isolation, quantitation, and estimation of the radioactivity in the glycosaminoglycans. The isolation procedure of the glycosaminoglycans from serum-containing media has recently been described in detail elsewhere (16), as has the separation and quantitation procedures using an Alcian blue dye method after electrophoresis on cellulose acetate (14, 15). With these methods recovery efficiencies of as little as 5 μg of hyaluronic acid are ~95%. In the experiments described here the addition of "carrier" hyaluronic acid (100 μg/ml of medium) did not appreciably increase recovery of the radioactive glycosaminoglycans in comparison with identical samples where no carrier had been added.

For the isolation of glycosaminoglycans from the cell surface (trypsin labile fraction) cultures were washed twice with PBS (12), and the cells were incubated with 2 ml of 0.125% trypsin solution for 1 h at 37°C. The cell suspension was centrifuged on a desk centrifuge, and the radioactive glycosaminoglycans were isolated from the supernate as described (16).

Quantitation of cell adhesion of synovial fibroblastic cells. In these experiments the routine trypsinization procedure, using 0.25% trypsin (Gibco Laboratories) was compared with trypsinization carried out with lower trypsin concentrations. Preliminary experiments indicated that a 0.08% trypsin solution (Gibco, diluted with PBS) was suitable for demonstrating differences in resistance to trypsinization between control fibroblastic cultures and cultures whose media were supplemented with supernates from mitogen-stimulated lymphocytes. All of these experiments were performed in 35-mm dishes.

The media were removed and the cultures were washed twice with 2.0 ml of PBS at 37°C. The trypsin solutions were then added (2.0 ml) and the dishes incubated in the CO_2 incubator for given time periods. For obtaining the total cell counts, the PBS-washed cells were incubated in 0.25% trypsin for 10–15 min at 37°C and detachment of all of the cells on the plate was confirmed visually using an inverted microscope. For the experiments with 0.08% trypsin, at the end of each incubation period the plates were gently angled 10 times from side to side so that the detached cells would be suspended. The suspension was carefully pipetted off and counted, as described above. The remaining attached cells were then detached, using 0.25% trypsin and the procedure described above, and enumerated. Recoveries of the combined cell numbers using 0.08% trypsin followed by the 0.25% trypsin procedures were ~90% compared with using the 0.25% trypsin procedure directly (see also Fig. 4).

RESULTS

In initial experiments synovial fibroblast cultures were established at low plating densities in 35-mm dishes (10,000 cells per dish) and after being allowed to attach for 24 h they were refed with fresh, nonconditioned medium containing 20% serum. 48 h later they were refed again with medium containing 20% serum and supplemented with the various conditioned media. The cells were then enumerated at given time points. It was observed that media from lymphocytes that had been stimulated with PHA or Con A had suppressive effects on the growth of synovial fibroblastic cells, while media from nonstimulated lymphocytes had generally slightly stimulatory effects. Neither Con A nor PHA added directly to the synovial fibroblast cultures at the same concentration that they were added to the lymphocytes had any significant effect on growth. However, it was noted in repeated experiments using the above feeding protocol that the suppressive effect on growth by the media from lectin-stimulated lymphocytes was not reproducible in all instances. If the cells were refed with conditioned media 1 d after they were allowed to attach in nonconditioned serum-containing medium, then the growth suppressive effect of media from lectin-stimulated lymphocytes was entirely reproducible in all experiments carried out for any one fibroblastic line. This feeding protocol was used in the experiments reported below. It was also noted, using this protocol, that synovial fibroblastic lines derived either from rheumatoid or nonrheumatoid synovia showed decreased growth when exposed to media from lectin-stimulated lymphocytes. However, lines that demonstrated consistently relatively short doubling times during several subcultures, also best illustrated the growth-suppressive effects of media from stimulated lymphocytes. The experiments reported here have been carried out with two relatively rapidly growing lines, the rheumatoid lines A.R. and L.D., and a relatively slowly growing line, L.W. The source of lymphocytes from normal individuals as well as patients with rheumatoid arthritis were able to generate similar suppressive activity for the growth of synovial fibroblasts. Although the amount of this activity has not been quantitated with respect to donor populations for the study, the lymphocytes of two normal donors, T.A. and P.A., were routinely used.

The effect of the inhibitory activity on the growth kinetics of human synovial fibroblasts is illustrated in Fig. 1. The fibroblastic cells were allowed to grow until the controls achieved their maximal saturation densities, with stable counts being reached between 6 and 11 d for the controls. Cultures that were sup-

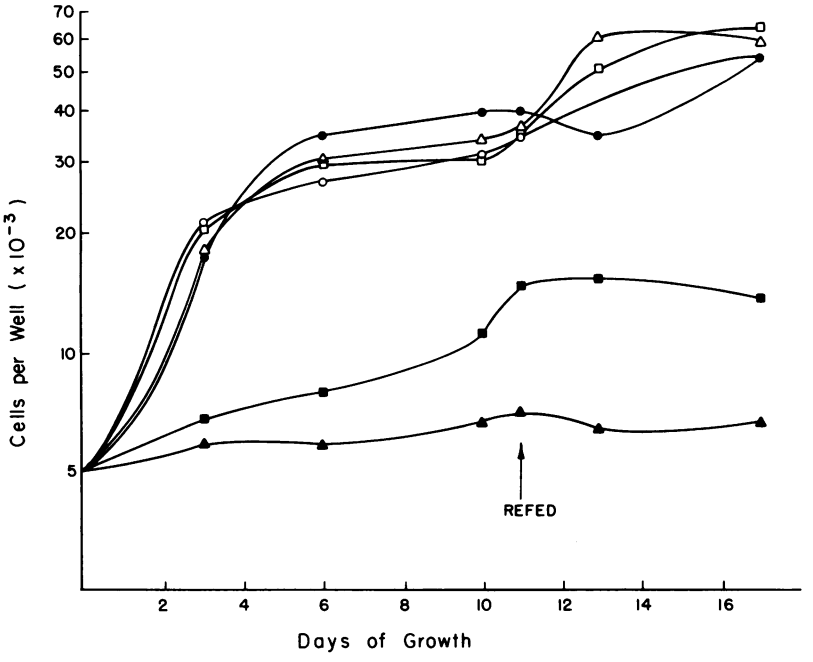

FIGURE 1 The effect of conditioned media and of refeeding with fresh serum on the growth kinetics of synovial fibroblastic cells. The growth kinetic protocol and preparation of conditioned media is given in Methods. Designations in the figure are as follows: NS-LM (●), PHA-LM (■), and Con A-LM (▲) indicate cultures fed with fresh media supplemented with conditioned media from cultures of nonstimulated lymphocytes, PHA-stimulated lymphocytes and Con A-stimulated lymphocytes, respectively. SFM-control (○), PHA-control (□), and Con A-control (△) indicate cultures supplemented with unconditioned serum-free medium or the identical serum-free medium containing the same final concentrations of PHA or Con A, as in the PHA-LM and Con A-LM supernates, respectively (also see text for other controls). The cell numbers of individual cultures were derived by enumerating the cells in two aliquots of the cell suspension from each dish. Each point shown represents the mean cell count from three dishes that were plated simultaneously. On day 11, when the control cultures had reached confluency, all cultures were washed once with PBS and refed with fresh serum-containing (20%) medium that had not been conditioned.

plemented with media from lectin-stimulated lymphocytes achieve very low rates of growth during the entire growth period. In fact, cultures supplemented with media from Con A-stimulated lymphocytes do not grow substantially and stay at the initial plating density, whereas those from PHA-stimulated lymphocyte cultures proliferate only very slowly (approximately a doubling of the initial plating density during the first 10 d of culture and a subsequent small increase between days 10 and 11). This inhibitory effect on the growth kinetics of fibroblastic synovial cells by media from lectin-stimulated lymphocytes was seen consistently in all 10 experiments performed using different synovial fibroblastic lines and lymphocytes from different donors.

Fig. 1 also illustrates the effect of refeeding of the cultures with 20% serum. The refeeding was carried out on day 11 after all of the controls had apparently reached their final saturation density. Fresh, serum-supplemented medium had no stimulatory effect on fibroblasts that were initially fed with medium supplemented with supernate from lymphocytes that had been stimulated with Con A. Thus, fresh serum-containing medium failed to stimulate the growth of these sparse cultures even though the initial conditioned medium from Con A-stimulated lymphocytes was removed and the cells washed before refeeding. The cells that were initially fed with medium from PHA-stimulated lymphocytes have grown very slowly through the first 11 d in culture, and refeeding these sparse cultures with fresh serum-containing medium also does not appear to have stimulated their growth (days 11–17). The control cultures, although they had achieved visual confluency between days 5 and 11, did have a further growth response to fresh serum-containing medium, with their saturation densities

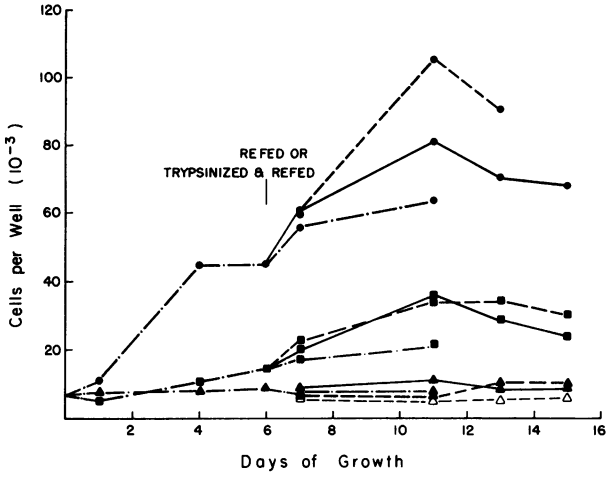

FIGURE 2 The effect of refeeding with fresh serum and of trypsinization on the growth of synovial fibroblasts fed initially with conditioned media. General protocol and designations are as in Fig. 1. On day 8 in this experiment, some wells from each group were either trypsinized and refed with nonconditioned serum-containing medium (short dashed lines) or just refed with this medium (solid lines). Some wells from each group were also allowed to continue past day 8 without any manipulation (short and long dashed lines). The trypsinization was of sufficient duration to cause the cells to start rounding, but not detach completely, as described in the text. Cells exposed to media supplemented with supernates from lymphocytes that had been in contact with Con A required twice as long to round in the presence of trypsin as the controls (text). The cells per well are plotted on a linear scale and all of the controls are not shown for the sake of clarity. Serum-free controls followed a pattern very similar to NS-LM but with somewhat lower cell numbers at each point. The open triangles (short dashed lines) represent EDTA treatment and refeeding that was done on cells initially supplemented with supernates from lymphocytes in contact with Con A. ●, NS-LM; ■, PHA-LM; ▲, Con A-LM.

rising well above those achieved between days 5 and 11. Other controls (data not shown) included supplementation with fibroblast-conditioned medium and supplementation with unconditioned serum-containing medium (Methods). These cultures demonstrated growth kinetics and growth responses to refeeding with fresh medium that were essentially identical to those of the controls shown in Fig. 1.

Supernates from lymphocyte-rich preparations that had been separated into nonadherent lymphocytes and mononuclear cells that included adherent cells (Methods) were also tested for their effect on the growth of synovial cells. It was found that supernates from both preparations suppressed growth to approximately the same extent after stimulation with Con A, following the protocol shown in Fig. 1 (data not shown). In the experiments described below, the supernates from the whole lymphocyte-rich preparation (including the adherent cells) were used as the conditioned media, as indicated in Methods.

Fig. 2 examines the effects of refeeding, trypsinization, and EDTA treatment on the growth kinetics of fibroblastic lines exposed to media from lectin-stimulated lymphocytes and controls. In this experiment each culture that was initially exposed to conditioned or control media has been either refed with fresh, nonconditioned serum-containing medium or exposed to a procedure that temporarily detached the cells from the plastic dish and then refed with the fresh serum-containing medium on day 6. Control cultures that were neither refed nor trypsinized, but allowed to stay in their original medium, proliferated only modestly past the 6-d time point until they reached their final saturation density. Refeeding of the control cultures with serum-containing medium resulted again in a stimulus to cellular proliferation and in higher final saturation densities. The brief trypsinization procedure followed by refeeding resulted in a further

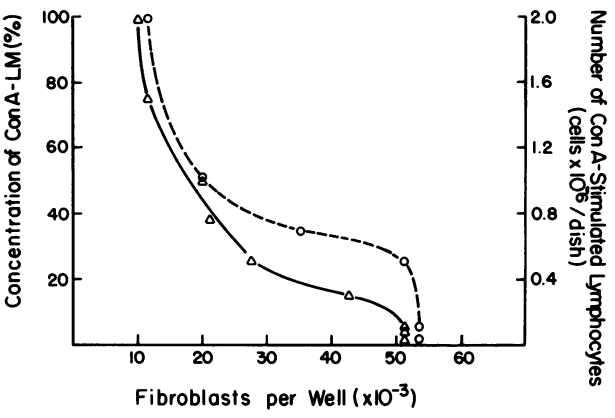

FIGURE 3 The effect of dilution of conditioned media from a fixed number of Con A-stimulated lymphocytes and of conditioned media from varying numbers of Con A-stimulated lymphocytes on synovial fibroblast growth. Two million lymphocytes in 2.0 ml of medium were exposed to Con A in 35-mm dishes as described under Methods. The concentration of this conditioned medium from Con A-stimulated lymphocytes (Con A-LM) is designated as 100% in the left hand ordinate of the figure. This medium was then diluted, using 10% serum-containing medium, to the concentrations shown expressed as a percentage of the original, undiluted medium. Aliquots of each dilution were assayed for growth-inhibitory activity on the synovial fibroblastic cells following the protocol described in Fig. 1 and in Methods (△). To study the effect of the lectin on the ability of different numbers of lymphocytes to produce growth-inhibitory activity, the number of lymphocytes indicated in the right hand ordinate of the figure was exposed to Con A in 35-mm dishes, as described under Methods. Aliquots from these conditioned media, derived from the different numbers of Con A-stimulated lymphocytes, were then used undiluted in the assay for growth-inhibitory activity on the synovial fibroblastic cells, as indicated above (○). The number of synovial fibroblastic cells at the end of the growth period are shown in the abscissa of the figure for both experiments. Each experimental point was derived from the mean of three observations.

enhancement of cellular proliferation of control cultures. However, fibroblastic cultures grown in the presence of media supplemented with supernates from Con A-stimulated lymphocytes show no proliferation under any of the protocols used, including trypsin or EDTA treatment. The cultures grown in the presence of supernates from PHA-stimulated lymphocytes grow very slowly and trypsinization does not produce any additional enhancing effect over refeeding on fibroblast proliferation.

In a similar experiment to that shown in Fig. 2, the refeeding or trypsinization procedures were again carried out on day 6, but a second refeeding was carried out on day 14, and the growth was monitored to day 22. The results were similar to those shown in Fig. 2 except that a further increase in growth was noted after the control lines were refed. The fibroblastic cultures grown in the presence of supernate from lymphocytes stimulated with Con A or PHA showed no growth and only slight growth (approximately a doubling), respectively, during the 22-d experimental period (data not shown).

Growth-suppressive activity of media from lectin-stimulated lymphocytes was stable at −10°C or 4°C for at least 2 wk, and the activity was rapidly lost on heating between 60° and 70°C for 4 h. The growth-inhibitory activity was diluted out with increasing concentrations of serum-containing medium in a curvilinear fashion (Fig. 3). Progressively smaller numbers of Con A-stimulated lymphocytes yield progressively less growth-inhibitory activity, but the relationship of the number of stimulated lymphocytes to the elaboration of fibroblastic growth-inhibitory activity also appears to be complex (Fig. 3).

The activity is nondialyzable and is retained and not significantly adsorbed by subjecting the conditioned media to ultrafiltration, using a filter with a molecular weight cut-off of 10,000. Exhaustive dialysis of Con A-stimulated lymphocyte medium against serum-free medium did not result in any loss of growth-inhibiting activity, and gave data essentially identical to the ultrafiltration experiments. About 75% of the activity was lost irreversibly by exposing the conditioned media to glycine-HCl buffer, 0.02 M at pH 2.0 for 3 h at room temperature (data not shown).

Two additional observations were made during the growth studies of fibroblasts grown in the presence of the various conditioned media. First, fibroblasts grown in the presence of Con A- or PHA-stimulated lymphocyte media do not show any evidence of a cytotoxic effect such as a tendency to round off or detach from the plastic surface throughout the growth periods that were studied. Cells exposed to these media generally have a fibroblastic or polygonal appearance and distinct cell margins when viewed in unstained preparations by light microscopy (Nomarski

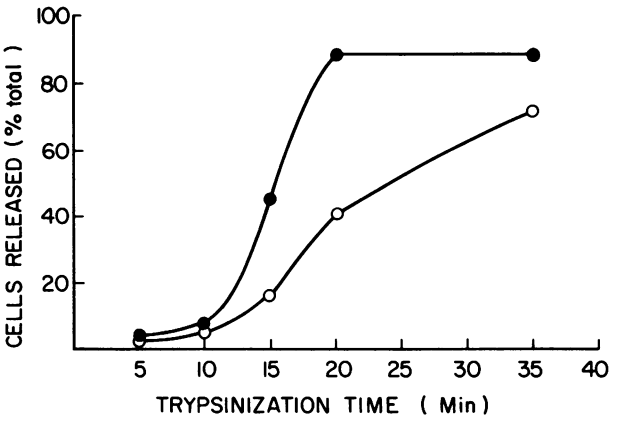

FIGURE 4 Time-course of trypsinization of synovial fibroblastic cells whose growth media were supplemented with conditioned media. Fibroblastic cells were seeded at 12,800 cells or at 18,000 cells per 35-mm dish. After the cells were allowed to attach for 24 h in serum-containing medium, the dishes with the lower seeding density were fed with SFM (●), and dishes with the higher seeding density were fed with Con A-LM. Under these conditions some growth also occurred in the Con A-LM-fed cells (○), so that reliable cell counts could be obtained for the shorter time points of the trypinization procedure. Mean total cell counts obtained by exposing some of the dishes to 0.25% trypsin, as described under Methods were 87,000 cells/dish for SFM and 59,000 cells/dish for the Con A-LM cultures. All other cultures were treated initially with 0.08% trypsin for the times shown in the abscissa of the figure, and then the remaining attached cells were removed with 0.25% trypsin following the procedure described under Methods. The number of cells released during any one time period by 0.08% trypsin are expressed as a percentage of the total number released by 0.25% trypsin, as shown in the ordinate of the figure. Designations are as in Fig. 1.

differential interference optics). Second, these cells are relatively resistant to removal from the plastic surface by trypsinization compared with the various controls. Routinely, control cultures could be effectively trypsinized in ~5 min at 37°C, using a 0.25% trypsin solution and the procedure outlined under Methods. Fibroblasts exposed to media from the mitogen-stimulated lymphocytes generally required 10–15 min under the same conditions before the cells rounded up and started detaching from the plate. If lower trypsin concentrations are used (for example 0.08% trypsin), then this difference in resistance to trypsinization can be quantitated (Fig. 4).

The effect of conditioned media on glycosaminoglycan synthesis by synovial fibroblastic cells is shown in Fig. 5. In this experiment, the fibroblasts were seeded at a low density (5,000 cells per 16-mm well), allowed to attach, and prelabeled the next day (day 1) with tritiated glucosamine and fresh medium as described in Methods. After 48 h fresh medium and label were added along with the supplements of the conditioned media. It can be seen that under these con-

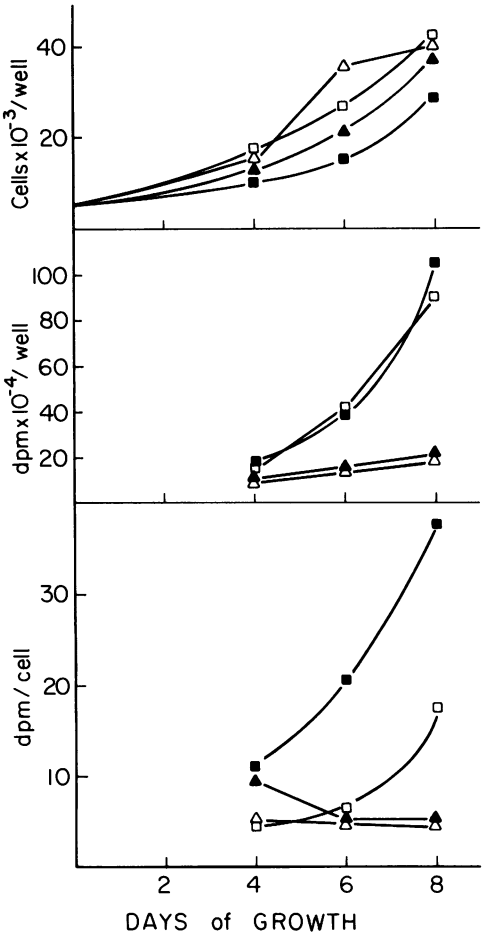

FIGURE 5 The effect of conditioned media on incorporation of radioactivity into synthesized by synovial fibroblastic cells plated at a low initial cell density. The feeding protocol and labeling procedure of the synthesized glycosaminoglycans secreted in the medium is described under Methods and in the text of Results. In this experiment all cultures were refed with fresh serum-containing medium and tritiated glucosamine (prelabeling period) on day 1. This was replaced on day 3 with media supplemented with tritiated glucosamine and the various conditioned media, as described in the text. The growth curves, total glycosaminoglycan secretion, and glycosaminoglycan secretion per cell are shown serially from top to bottom. The designations SFM (△), CFM (▲), NS-LM (□), and PHA-LM (■) are explained in the legend to Fig. 1 and in Methods.

ditions, the rate of incorporation of radioactivity into secreted glycosaminoglycans per cell is considerably higher for synovial cells exposed to PHA-stimulated lymphocyte medium compared with non-stimulated lymphocyte medium. It should be noted that this feeding protocol is different from the ones used to illustrate the maximum effects of the conditioned media on growth (Figs. 1–3) in that all cultures here are allowed to proceed through to day 3, in the absence of conditioned media. Under these conditions, differences in growth between cells exposed to supplementations of media from mitogen-stimulated lymphocytes and control cells tend to be minimized. Similar data (not shown) were obtained using media from Con A-stimulated lymphocyte cultures.

However, the rate of glycosaminoglycan synthesis by fibroblastic cells is also influenced by the particular experimental protocol that is used, since the rate of secretion of glycosaminoglycans can also be related to the cell density with some cell lines (32). In the experiment shown in Fig. 6, the cells were plated at three different cell densities, prelabeled for 3 d, and then exposed to the various control and conditioned media in the presence of fresh label for an additional 2 d. The radioactivity incorporated into the glycosaminoglycans was quantitated during this period of exposure to the conditioned and control media. Under these conditions, cultures fed with PHA-stimulated lymphocyte medium incorporate more radioactivity into secreted glycosaminoglycan per cell than cultures fed with nonstimulated lymphocyte medium, which in turn incorporate more radioactivity than the control cultures supplemented with serum-free medium. The differences in incorporation of radioactivity into glycosaminoglycan secretion per cell are most pronounced under conditions that also demonstrate the greatest differences in final cell numbers (e.g., compare 10,000 cell-plating density with the 100,000 cell-plating density experiment).

The effect of media from lectin-stimulated lymphocytes on glycosaminoglycan production by the synovial cells was further examined using the micro method we have described for quantitation of glycosaminoglycans from cultured cells (14, 15). With this method it was possible to obtain reliable specific activities and calculate net rates of synthesis for secreted hyaluronic acid in the medium (16). Other glycosaminoglycans and cell surface hyaluronic acid were not present in sufficient amounts to permit reliable quantitation of weight at lower cell densities.

Fig. 7 illustrates the data for fibroblastic cells that had been plated at the same initial plating density following the same protocol as for the growth experiments (Figs. 1 and 2), (i.e., no prelabeling period with normal serum-containing medium). There was an ~4.5-fold increase in incorporation of radioactivity into hyaluronic acid per cell that had been exposed to Con A lymphocyte medium compared with the controls over a 3-d labeling period. Approximately the same relative increase in hyaluronic acid net synthesis was noted in the media fractions (bottom right hand panel). The specific activities of medium hyaluronic acid of the controls were very similar to those obtained from cultures treated with media from Con A-stimulated lymphocytes (~1,500 dpm/μg hyaluronic acid), suggesting that the labeled glucosamine had been

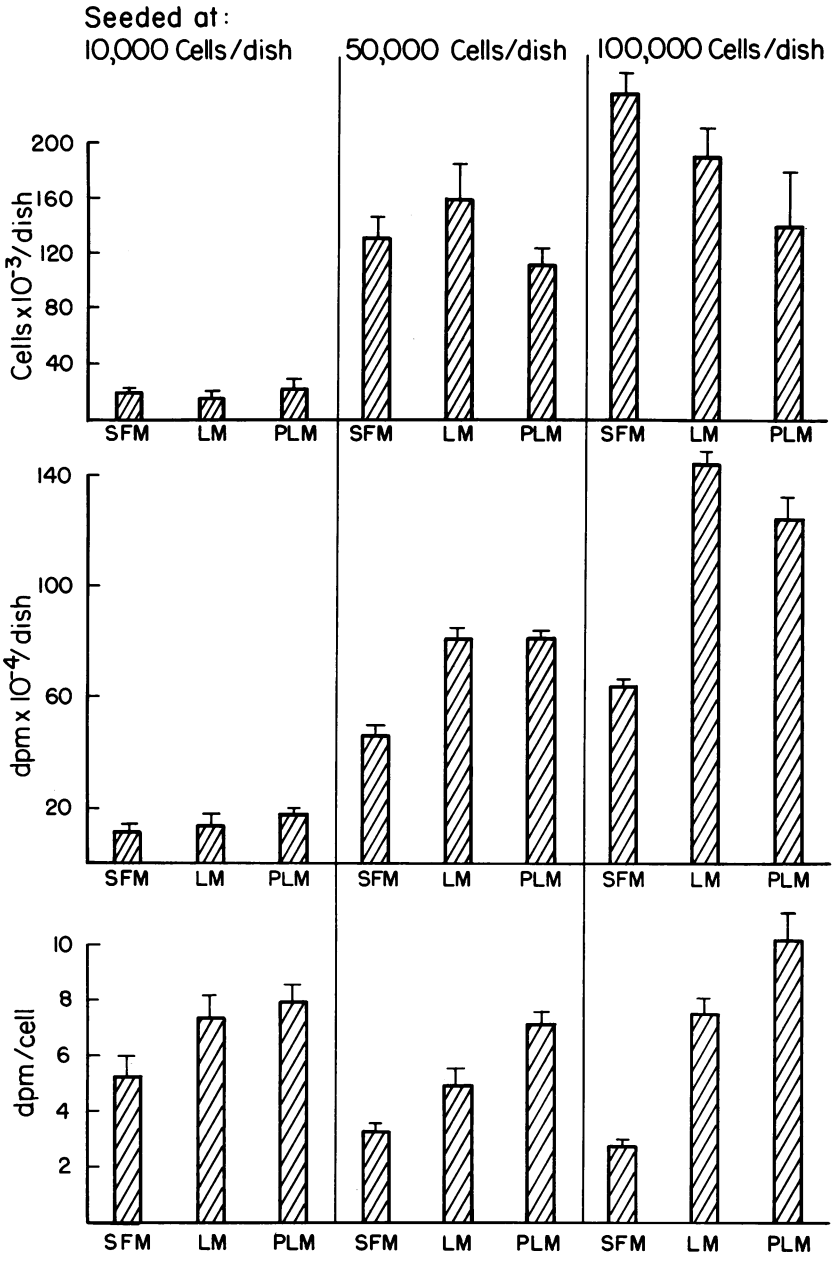

FIGURE 6 The effect of conditioned media on incorporation of radioactivity into glycosaminoglycan synthesized by synovial fibroblastic cells plated at different cell densities. The synovial fibroblastic cells were plated at 10,000, 50,000, and 100,000 cells per 35-mm dish. The labeled glycosaminoglycans from the medium were isolated for a 2-d labeling period that followed a 3-d prelabeling period as explained in the text. Designations are as in Fig. 1, except that the abreviations LM and PLM are used instead of NS-LM and PHA-LM, respectively. Each hatched bar represents the mean of three experiments and the range from the mean is indicated by the height of the T-bar.

diluted to about the same extent by endogenously synthesized glucosamine, presumably largely derived from medium glucose. Incorporation of radioactivity into the cell surface fraction was also markedly stimulated in fibroblasts exposed to Con A-stimulated lymphocyte media compared with controls. It will be noted, however, that with this feeding protocol, there was not significant growth of the cells treated with media from Con A-stimulated lymphocytes, while the control cultures grew as expected. However, we had

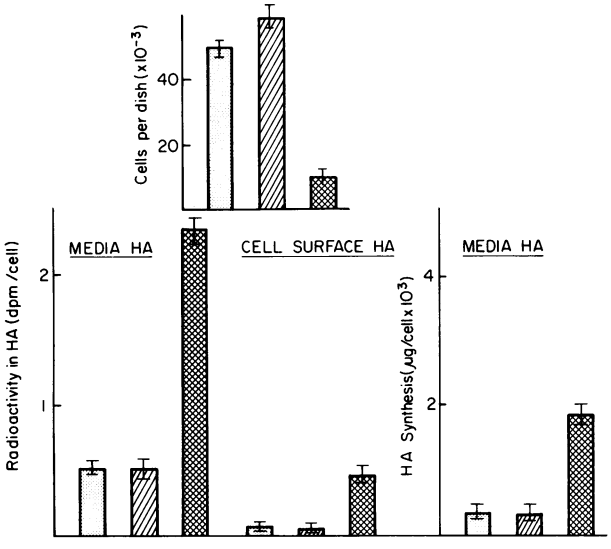

FIGURE 7 The effect of conditioned media on net glycosaminoglycan synthesis by synovial fibroblastic cells that have achieved different final cell densities. Cells were plated at ~14,000 cells per dish and allowed to attach for 24 h in serum-containing medium, they were fed with similar fresh medium containing 0.5. μCi of [³H]glucosamine per ml and supplemented with the appropriate conditioned media. After 3 d the media were removed and the media and cell surface glycosaminoglycans were isolated. In the media fractions both the incorporation of radioactivity (bottom, left hand panel) and the net rates of synthesis were estimated (bottom, right hand panel), while in the cell surface fraction (bottom middle panel) only the incorporation of radioactivity into the isolated hyaluronic acid (HA) was estimated. The top panel shows the final cell densities achieved at the end of the experiment. Strippled bars-control cultures supplemented with serum-containing medium; hatched bars-control cultures supplemented with nonstimulated lymphocyte medium; crosshatched bars = cultures supplemented with Con A-stimulated lymphocytes medium. There were three experiments for each bar and the means and ranges are indicated. HA = hyaluronic acid.

previously shown that, with at least certain cell types, there is a rapid decrease in net hyaluronic acid synthesis with increasing cell density (32). An experiment was therefore designed to minimize the cell density effect (Fig. 8).

This experiment was carried out with a more slowly growing line than the one used in Fig. 7. 5 d was required for the control cultures that were plated at relatively low cell densities to reach the number of the growth-arrested fibroblasts that were plated at relatively high cell densities and exposed to Con A-stimulated lymphocyte media (legend to Fig. 8). The slowly growing line in this experiment was chosen to minimize the number of cell cycles of the control cultures over a relatively prolonged period of glycosaminoglycan synthesis and secretion. It can be seen that even after a 5-d period, the amount of radioactivity per cell that has accumulated into the hyaluronic acid synthesized by fibroblasts exposed to Con A-stimulated-lymphocyte medium is about twice what it is in the controls (at approximately the same final cell densities). Net hyaluronic acid synthesis, however, is only ~30% higher in cultures exposed to Con-A-stimulated media compared with controls (bottom right hand panel, Fig. 8). This is probably explained by the higher specific activity of the hyaluronic acid from the cultures exposed to Con A-stimulated lymphocyte media, compared with controls (2,800 dpm/μg compared with 2,100 dpm/μg), suggesting that labeled glucosamine had been diluted less in cultures exposed to Con A-stimulated lymphocyte media than in control cultures.

DISCUSSION

The experiments described suggest the elaboration of a soluble factor by human peripheral blood lymphocytes exposed to lectins, which inhibits the proliferation of synovial fibroblastic cells. Growth-inhibitory

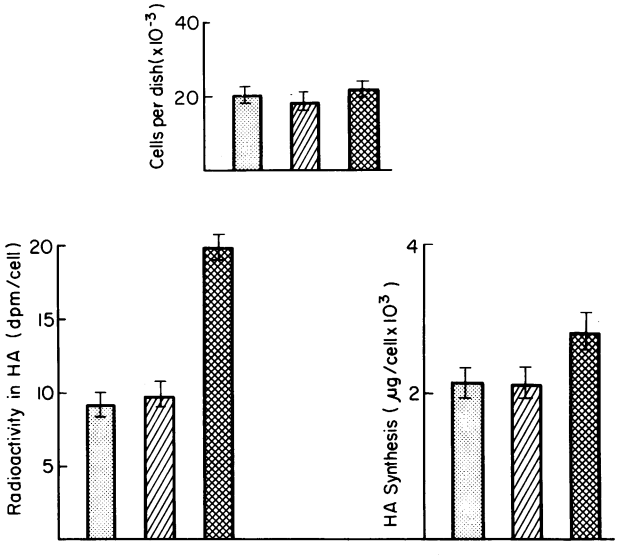

FIGURE 8 The effect of conditioned media on net glycosaminoglycan synthesis by synovial fibroblastic cells that have achieved the same final cell density. Synovial fibroblastic cells were plated at 10,000 cells per dish and at 23,000 cells per dish. The protocol was similar to that described for Fig. 8, except that control media (serum-free and nonstimulated lymphocyte media) were used in the feeding of dishes plated at the lower initial plating density and Con A-stimulated lymphocyte media were used in the feeding of dishes plated at the higher initial plating density. 1 μCi/ml of medium was used in this experiment, as described under Methods. When control cultures in adjacent wells had achieved approximately the same cell density as the cultures fed with media supplemented with Con A-stimulated lymphocyte medium, the experiment was terminated and the hyaluronic acid (HA) was isolated from the media. Designations are as those for Fig. 8. There were four experiments done for each group of cultures.

activity is not detected if the lymphocytes are not exposed to lectins and that the lectins themselves cannot account for the inhibitory effect on growth (Fig. 1). Supernates from the lymphocytes themselves, in fact, tend to have a small degree of growth-stimulatory activity over other controls in the protocol used (Fig. 1 and other data not shown). Small increases in the proliferation of synovial cells supplemented with nonstimulated lymphocyte supernates have been noted before (17), although "connective tissue-activating peptide 1" (11) apparently does not stimulate DNA synthesis of fibroblasts.

Many types of cytotoxicity that require contact of the lymphocyte with the target cell have been reported and these have been reviewed (18–20). Cytotoxicity of soluble mediators released from lymphocytes has also been studied in a number of laboratories (20), and the kinetics of release of "lymphotoxin" following PHA stimulation have been described (21). Human lymphotoxin was initially partially purified and characterized as a large molecular weight protein (22) and more recently has been shown to consist of cytotoxically active fractions with molecular weights of 75,000 and 45,000 (23), but smaller molecular weight components have also been identified with cytotoxic activity. Cytotoxicity of these proteins was assessed generally by cell death or chromium release from mouse L cells (23, 24). A proliferation-inhibition factor has been described using the uptake of [^{3}H]thymidine into HeLa cells (25–27), but this factor does not seem to affect the proliferation of L cells or the proliferation of chick embryo fibroblasts. There is some evidence to suggest that lymphotoxin and the proliferation inhibition factor may represent similar or identical substances that have been assayed in different concentrations and in different systems (28–31), but is distinct from a factor that inhibits DNA synthesis of mitogen-stimulated lymphoid cells or L cells (29).

The factor activity that we describe does not have cytotoxic action towards synovial fibroblastic cells, but markedly arrests their growth, if added before active replication of the cells is initiated during the log phase of growth. It is not clear at this time whether the growth-inhibitory factor for synovial cells represents the same or different molecular species with respect to the cytotoxic lymphotoxins that have been described as indicated above. However, this type of growth-inhibitory activity for human synovial fibroblastic cells has not been described.

Of particular interest is the analysis of the growth kinetics of the synovial fibroblastic cells exposed to the different conditioned media. Cultures exposed to supernates from the mitogen-stimulated lymphocytes fail to respond to the stimulatory effect of serum. This is well seen in Figs. 1 and 2. In Fig. 1 a very slow rate of growth is observed in synovial cells that were in contact with medium from PHA-stimulated lymphocytes, but this appears to be independent of refeeding with serum and in the cultures that were in contact with medium from Con A-stimulated lymphocytes, no proliferative response is observed throughout the growth period.

It should be emphasized that the increased adherence to the culture dish of cells exposed to media from mitogen-treated lymphocytes (Fig. 5) argues against a cytotoxic effect by such media. Instead it suggests alterations (possibly in the biosynthesis) of membrane components that are also of a long term or persistent nature.

The data in Figs. 5–8 indicate that the plant mitogens PHA and Con A cause the lymphocytes to secrete a product(s) that can stimulate glycosaminoglycan synthesis by synovial fibroblastic cells (on a per cell basis), over and above any stimulatory activity secreted by lymphocytes in the absence of the plant mitogens. The latter, however, does contain some activity that can stimulate incorporation of radioactivity into glycosaminoglycans over other controls (Fig. 6), and is probably related at least in part to connective tissue-activating peptide I activity, whose elaboration is not affected by mitogenic stimulation of lymphocytes (11). The additional increment in glycosaminoglycan synthesis by synovial fibroblastic cells exposed to media from lymphocytes stimulated with Con A or PHA is of some interest in that this activity appears to be plant mitogen dependent and cannot be entirely explained as a secondary effect related to the cell density of the culture (Fig. 8), (32).

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