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Effect of Macrophages on Fibroblast DNA Synthesis and Proliferation[1] (41373)

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Abstract. Mouse peritoneal macrophages incubated with mouse 3T3 fibroblasts modulate DNA synthesis by the fibroblasts as measured by ^{125}IUDR incorporation into DNA. The factor responsible for this activity is soluble and inhibits fibroblast DNA synthesis when present in high concentrations, whereas stimulation was observed at lower concentrations. Similar findings were observed when soluble fractions from human peripheral monocytes were incubated with primary human skin fibroblasts. In addition to increased DNA synthesis, there was a significant increase in human fibroblast proliferation as measured by direct cell counting. These findings support the hypothesis that macrophages play a regulatory role in wound healing through their capacity for positive and negative regulation of fibroblast DNA synthesis and proliferation.

The factors responsible for fibroblast migration and proliferation during wound repair remain unclear. Some early studies suggested that various blood cells could become transformed into fibroblasts at wound sites (1–5), but subsequent parabiotic studies demonstrated that hematogenous precursors were not transformed into wound fibroblasts (6). Of late, attention has been drawn to the role of cellular interactions and factors produced by one cell type during wound healing that can modulate the activity of a target cell. For example, Johnson and Ziff (7), Postlethwaite *et al.* (8), and Wahl *et al.* (9) have described factors produced by lymphocytes (lymphokines) which stimulate fibroblast migration, proliferation, and perhaps collagen synthesis.

In 1961, histologic studies by Ross and Benditt demonstrated the early appearance of polymorphonuclear neutrophils and macrophages at the site of open guinea pig wounds prior to the appearance of the maximal level of fibroblasts (10). These observations were consistent with the concept that the neutrophil provided signals needed for subsequent fibroplasia (11–13). However, this concept was proven inaccurate because animals made neutropenic by administration of antineutrophil serum had normal wound healing in the absence of neutrophils (14). In contrast to the neutrophil studies, guinea pigs made monocytopenic by administration of hydrocortisone and antimacrophage serum showed significant delays in fibroblast proliferation within the wounds (15).

The initial *in vivo* observations suggesting a role for macrophages in wound healing have been extended to cell culture studies where a "macrophage-dependent fibroblast stimulating activity" (M-FSA) has been defined (16). Recently, Leibovich (17) and Martin *et al.* (18) described a macrophage-derived factor responsible for fibroblast DNA synthesis and cell division. However, in these studies the fibroblast growth rate was reduced by serum deprivation prior to the analysis of the macrophage-derived factors, thus making it difficult to differentiate replacement of normal serum constituents from regulatory events. In a report by DeLustro *et al.* (19), a nondialyzable, soluble factor from human peripheral blood monocytes was described which promoted human foreskin fibroblast growth. The fibroblast growth-stimulating activity was demonstrated in the presence of 10% fetal calf serum and was not inhibited by indomethacin, thus suggesting that the effect was not mediated by prostaglandin.

In the present studies, we demonstrate that direct cell contact, at appropriate cell-to-cell ratios, between mouse peritone-

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al inflammatory cells (mainly macrophages) and fibroblasts modulates fibroblast DNA synthesis. The factor responsible for this activity is soluble and can be demonstrated in the presence of 10% fetal calf serum. Likewise, human macrophage-conditioned medium stimulated human dermal fibroblast DNA synthesis and proliferation. These findings support the hypothesis that the macrophage is a key cell capable of modulating fibroblast proliferation during wound repair.

Materials and Methods. *Fibroblast cultures.* Swiss albino mouse fibroblasts (3T3, CCL-92; American Type Culture Collection, Rockville, Md.) were maintained in Eagle's minimal essential medium (410-1200, GIBCO, Grand Island, N.Y.) containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 m*M* Tricine buffer (pH 7.4). The cultures were plated at low density to ensure the maintenance of density-dependent growth inhibition characteristics of 3T3 fibroblasts. The fibroblast cultures were routinely passed by trypsinization (0.1%) and cells between passage 125 and 140 were used in the present studies.

In one series of studies, a primary line of human dermal fibroblasts in the ninth passage was used to determine the interaction of human peripheral macrophages and conditioned medium on fibroblast DNA synthesis and proliferation.

Preparation of mouse peritoneal exudate cells. Inflammatory cells were harvested from the peritoneal cavity of female Swiss albino mice by washing the cavity with 8 ml of serum-free Eagle's medium. The peritoneal cells were harvested 5 days after intraperitoneal injection of 0.2 ml 10% Brewer thioglycollate (DIFCO Laboratories, Detroit, Mich.). Cells from several mice were pooled, lightly centrifuged (50*g*) to remove platelets, and then washed three additional times (1000*g*, 10 min) in serum-free Eagle's medium. The cells were suspended in Eagle's medium with 10% fetal calf serum and added to 24-hr mouse 3T3 fibroblast cultures. Preparation of peritoneal exudate cells by this procedure resulted in a population of inflammatory cells consisting of greater than 70% macrophages as determined by morphology, glass adherence, latex phagocytosis, and nonspecific esterase staining.

Preparation of mouse peritoneal macrophage-conditioned medium. Ten-milliliter aliquots of the peritoneal exudate cells (5×10^5/ml) were placed in 100-mm-diameter tissue culture dishes. Loosely adherent cells were removed and the attached, macrophage-enriched population of cells was incubated for 48 hr with Eagle's medium containing 10% fetal calf serum. After incubation, the medium was removed and centrifuged at 1200*g* to remove any suspended cells. The conditioned culture fluid was aliquoted and frozen at −20° until assayed with mouse 3T3 fibroblasts for alteration of DNA synthesis.

Measurement of mouse 3T3 and human fibroblast DNA synthesis by incorporation of ^{125}IUDR. The mouse 3T3 fibroblasts were adjusted to $2.5-5.0 \times 10^4$ cells/ml in Eagle's medium containing 10% fetal calf serum and 0.2 ml was pipetted into wells of microtest plates (Falcon No. 3040) and incubated at 37° for 24 hr. The medium was then removed and replaced with 0.2 ml of Eagle's medium with 10% fetal calf serum or medium containing various concentrations of peritoneal inflammatory cells (macrophage-enriched) or peritoneal macrophage-conditioned medium. The plates were incubated for an additional 24 or 48 hr, the medium was removed, and then incubated for 2 hr with 0.2 ml Eagle's medium containing 10% fetal calf serum and 0.5 μCi/ml of ^{125}IUDR (2000 Ci/mmol, New England Nuclear, Boston, Mass.). The plates were washed three times with serum-free Eagle's medium, fixed with 70% methanol, and sprayed with clear plastic (Colony Paint Co., Baltimore, Md.). The individual wells were cut out with a band saw and counted in a deep-well gamma scintillation counter to determine the amount of ^{125}IUDR incorporated into the 3T3 fibroblast DNA (20). In one series of studies, 48-hr human monocyte-conditioned medium was added at various concentrations to human dermal fibroblasts. After an additional 48-hr incubation, DNA synthesis was measured by the ^{125}IUDR incorporation method as described above.

To ensure that the ^{125}IUDR was actually incorporated into DNA, 10^4 3T3 mouse fibroblasts were incubated with 10^2 peritoneal inflammatory cells for 48 hr and then pulse labeled with ^{125}IUDR as above. The fibroblast cell layer was then washed three times with serum-free Eagle's medium (4°), the cells were lysed by sonication and DNA was precipitated by the addition of trichloroacetic acid (5%) at 4°. The precipitated DNA was washed three times and then the DNA was extracted with 5% trichloroacetic acid at 90° for 15 min, and the amount of incorporated isotope was determined (21).

Preparation of human peripheral monocytes. Approximately 60 ml of human peripheral blood was obtained by venipuncture in a heparinized syringe. Cells were removed by centrifugation (800*g*, 20 min) and the plasma was aspirated. The white cells were removed from the buffy coat and diluted 1 to 5 with serum-free Eagle's medium. The white cell suspension was layered onto Ficoll–Hypaque (Pharmacia) and centrifuged (400*g*, 35 min). The cells at the interface were removed, suspended in fresh Eagle's medium, centrifuged (300*g*, 12 min) and washed twice more (200*g*, 10 min). The pelleted cells were then resuspended in 5 ml of barbitol buffer (4 m*M*, pH 7.2) and washed three times (50*g*, 10 min) to remove platelets. The leucocytes were resuspended in Eagle's medium containing 0.1% fetal calf serum, counted, and plated at a density of 2.5×10^6/100-mm tissue culture plate. Macrophages were allowed to adhere for one hour at 37° and nonadherent cells (lymphocytes) were removed by four vigorous washings with serum-free Eagle's medium. Greater than 96% of the glass-adherent cells had characteristic macrophage morphology, were nonspecific esterase positive, and phagocytized latex particles. The macrophages were incubated for 48 hr in Eagle's medium containing 10% fetal calf serum. The human macrophage-conditioned medium was collected, centrifuged at 1000*g* to remove any floating cells and then added at various dilutions to human skin fibroblast cultures (ninth passage) to test for fibroblast DNA synthesis (^{125}IUDR incorporation) and cell proliferation by trypsinization and direct cell counting in a hemocytometer.

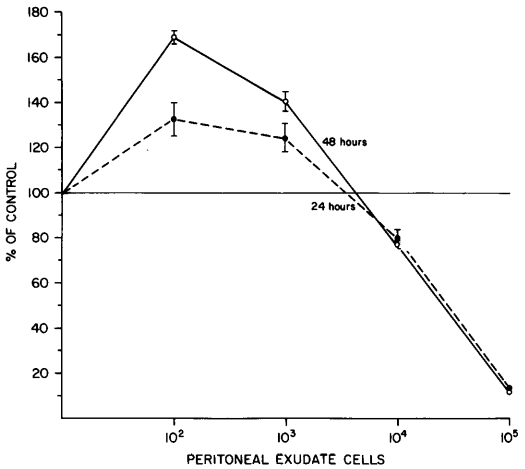

FIG. 1. Effect of mouse peritoneal inflammatory cells on 3T3 mouse fibroblast DNA synthesis. Peritoneal cells were cultured with 24-hr 3T3 fibroblasts (subconfluent) for an additional 24 to 48 hr prior to the addition of ^{125}IUDR for 2 hr to measure 3T3 fibroblast DNA synthesis. Data are expressed relative to 3T3 fibroblast cultures incubated with Eagle's medium containing 10% fetal calf serum (100%) and each point represents the mean of six observations.

Measurement of phospholipase A activity. Phospholipase A activity in peritoneal macrophage-conditioned medium was measured by the procedure described by Franson *et al.* (22) using [1-^{14}C]oleate-labeled *E. coli* phospholipid as substrate.

Statistical analysis of data. Student's *t* test was used for statistical analyses.

Results. *Modulation of 3T3 fibroblast DNA synthesis by peritoneal inflammatory cells.* When 10^2 to 10^3 peritoneal exudate cells were added to 24-hr 3T3 mouse fibroblast cultures, there was a significant stimulation of ^{125}IUDR incorporation into 3T3 fibroblast DNA at 24 and 48 hr (Fig. 1). When a ratio of peritoneal exudate cells to 3T3 mouse fibroblasts was 1 to 1 or greater, there was a significant inhibition of ^{125}IUDR incorporation into 3T3 mouse fibroblast DNA (Fig. 1).

The ^{125}IUDR was specifically incorporated into fibroblast DNA as verified by

characteristic chemical isolation (21); greater than 95% of the fibroblast-associated radioactivity was demonstrated in DNA. Peritoneal exudate cells incubated alone did not incorporate ^{125}IUDR into DNA.

Effect of peritoneal macrophage-conditioned medium on 3T3 mouse fibroblast DNA synthesis. The peritoneal macrophage factor which modulates 3T3 fibroblast DNA synthesis is soluble and could be detected in 48-hr conditioned medium (Fig. 2). Peritoneal macrophage-conditioned medium caused significant stimulation of fibroblast DNA synthesis when the medium was diluted 1:50 or 1:100 with Eagle's medium containing 10% fetal calf serum (Fig. 2). As the medium was diluted beyond 1:100, the activity returned to the control value of Eagle's medium containing 10% fetal calf serum (Fig. 2). When the macrophage-conditioned medium was tested at dilutions of 1:5 or more concentrated, there was inhibition of ^{125}IUDR incorporation into the fibroblast DNA.

The possibility that the increased ^{125}IUDR incorporation may be due to an alteration of 3T3 fibroblast membrane function is unlikely because the medium that altered fibroblast DNA synthesis was negative for phospholipase A activity (22). The presence of phospholipase A activity would have suggested that increased isotope incorporation was due to altered membrane permeability. Furthermore, all of these studies were carried out in the presence of 10% fetal calf serum, a potent inhibition of many proteolytic enzymes.

Effect of human macrophage medium on human fibroblast DNA synthesis. Incubation of subconfluent human dermal fibroblasts for 48 hr with conditioned medium obtained from human macrophages (48 hr) resulted in a marked stimulation of DNA synthesis as measured by ^{125}IUDR incorporation (Fig. 3). When the medium was diluted beyond 1:2, the stimulatory effect was diminished and by 1:500 was equal to the control value of fibroblasts incubated with fresh Eagle's medium containing 10% fetal calf serum (Fig. 3). The human macrophage

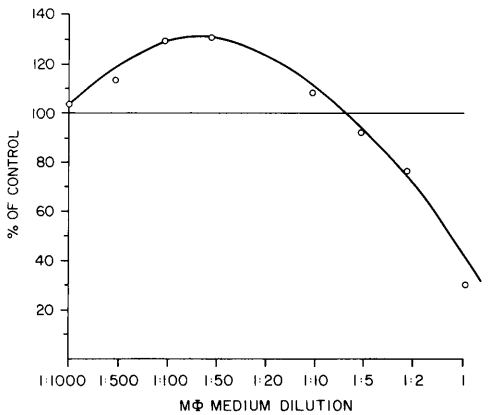

FIG. 2. Effect of mouse peritoneal macrophage medium on 3T3 fibroblast DNA synthesis. Various concentrations of 48-hr peritoneal macrophage-conditioned medium was added to 24-hr 3T3 fibroblasts (subconfluent) and the incubation was continued for an additional 48 hr prior to the addition of ^{125}IUDR for 2 hr. Each point represents the mean of five observations compared to control 3T3 fibroblast cultures incubated in Eagle's medium with 10% fetal calf serum (100%).

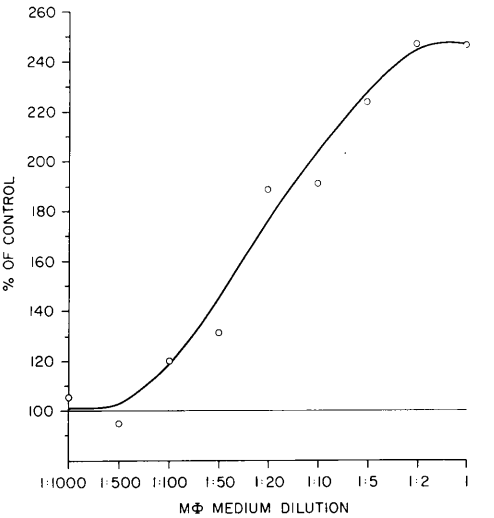

FIG. 3. Effect of human macrophage medium on human fibroblast DNA synthesis. Human dermal fibroblasts in the ninth passage were plated at a density of 10^4 cells per well in 96-well microtiter plates. After 24 hr, human macrophage-conditioned medium was added in various concentrations and following an additional 48-hr incubation, ^{125}IUDR was added for 2 hr. Each point is the mean of 10 observations and compared to control fibroblast cultures incubated in Eagle's medium with 10% fetal calf serum (100%).

TABLE I. EFFECT OF HUMAN MACROPHAGE MEDIUM ON HUMAN FIBROBLAST PROLIFERATION

Culture condition	Cells × 10^5 ± SEM	
	Day 0	Day 4
Control (n = 3)	1.25	2.45 ± 0.11
Macrophage medium[a] (n = 4)	1.25	3.95 ± 0.15*

[a] Human macrophage medium was diluted 1:5 with Eagle's medium containing 10% fetal calf serum.
*$P < 0.001$ compared to control at 4 days.

medium was not found to inhibit human fibroblast DNA synthesis. When the human fibroblasts were incubated for four days with the macrophage-conditioned medium (diluted 1:5), there was a significant increase in cell proliferation as determined by direct cell counting techniques (Table I).

Discussion. The histologic studies by Ross and Benditt showed that the influx of fibroblasts to the wound site is preceded by the appearance of macrophages (10). In further studies, Leibovich and Ross demonstrated that wounds depleted of macrophages "appeared immature, both in terms of the degree of debridement and extent of fibrosis" (15). Investigations by Hunt *et al.* have provided evidence that injection of endotoxin stimulated 21-day wound macrophages into rabbit corneas resulted in increased scar formation (23). In addition, Casey *et al.* (24) demonstrated that intradermal injection of allogenic macrophages into rat skin accelerated wound healing. All of these studies suggest that macrophages regulate fibroplasia during wound healing. The present studies demonstrate directly that interaction between mouse macrophage-rich peritoneal exudate cells and 3T3 fibroblasts results in modulation of 3T3 fibroblast DNA synthesis.

When a critical ratio of peritoneal inflammatory cells to fibroblasts was established, there was a significant stimulation of fibroblast DNA synthesis as measured by ^{125}IUDR incorporation (Fig. 1). As the number of peritoneal inflammatory cells was increased, cytostasis or inhibition of fibroblast DNA synthesis occurred (Fig. 1). In addition, the factor responsible for directing fibroblast DNA synthesis is soluble and is attributed to the macrophage (Figs. 2 and 3). Furthermore, soluble factors released from human macrophages stimulate cell division (Table I) in addition to stimulation of DNA synthesis. Cytostasis was not observed in the human macrophage–fibroblast system. Perhaps the factor produced by the "non-activated" human monocytes is not produced in concentrations high enough to cause dermal fibroblast cytostasis as was observed in the activated mouse macrophage system. Concentration differences may actually explain the altered responses because the density of mouse macrophages (Fig. 2) was greater than the density of the human monocytes (Fig. 3). Alternatively, the observed dissimilarity between the responses and the magnitude of the responses (Figs. 2 and 3) may be due to species differences. The mouse macrophage medium does not modulate human dermal fibroblast DNA synthesis (unpublished observation).

The possible relationship or similarity between the factors described in this report and other growth factors (25–27) has not been determined. In recent years, a number of macrophage factors in a variety of systems have been described and these factors have been shown to stimulate or suppress fibroblast proliferation and collagen synthesis depending upon the assay system (see review (28)). For example, the "macrophage-dependent fibroblast-stimulating activity" reported by other laboratories required culture conditions where fibroblast growth rates were reduced by serum deprivation (16–18). In contrast, the present studies are in agreement with the findings of DeLustro *et al.* (19) and demonstrate macrophage-dependent fibroblast-DNA-stimulating activity in the presence of nondialyzed, non-heat-inactivated 10% fetal calf serum. This suggests that the factor is active in addition to the normal serum growth-promoting factors. It is unlikely that the fibroblast growth-promoting factor released by the macrophages is fibronectin because of ready availability of this gylcoprotein present in the 10% fetal calf serum. Furthermore, this macrophage factor is not likely

to be a protease because all of the experiments were carried out in the presence of 10% fetal calf serum, a potent inhibition of most proteolytic enzymes. The absence of phospholipase A activity negates the possibility of altered membrane permeability induced by the macrophages.

At this time, it is not clear whether one macrophage product is responsible for both stimulation and inhibition of fibroblast proliferation or if multiple factors are present. Because the present studies were carried out in the presence of 10% fetal calf serum, it has not been possible to isolate and characterize the factor(s) responsible. Conceivably, one factor could elicit opposite responses depending upon the concentration of the factor. However, it is possible that the macrophage produces multiple factors which can modulate fibroblast chemotaxis, proliferation, and collagen synthesis.

The present studies did not determine whether factors responsible for alteration of fibroblast DNA synthesis and cell proliferation are produced by macrophages or are the result of conversion of existing factors or inhibitors in fetal calf serum. However, findings by Wahl *et al.* (29) suggest that macrophages can produce fibroblast stimulating factors in the absence of protein in the medium.

Findings to date are consistent with the hypothesis that macrophages contribute to the regulation of fibroplasia during wound repair. At the onset of repair, macrophages begin to debride the wound and, in turn, provide signals to stimulate fibroblast DNA synthesis and proliferation. When a critical ratio of macrophages to fibroblasts is established, then fibroblast proliferation is inhibited and the increased population of fibroblasts is established in the wound site to commence the repair process. Perhaps the inhibition of wound repair observed following steroid treatment is due to the anti-inflammatory effects on the macrophage population with subsequent inhibition of factors needed to enhance fibroplasia rather than a direct inhibition of fibroblast function by the steroids.

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