A Macrophage-Dependent Factor That Stimulates the Proliferation of Fibroblasts *In Vitro*

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Whole blood serum (HBS) stimulates the proliferation of fibroblasts *in vitro*, while platelet-poor plasma serum (PPPS) does not. Fibroblasts grown in the presence of PPPS are truly quiescent in that they are not deprived of nutrients in the culture medium and less than 3% of the cells synthesize DNA and divide. *In vivo* experiments have suggested that macrophages are necessary for stimulation of fibroplasia during wound repair. We have utilized the difference in growth-promoting activity between HBS and PPPS to study the ability of macrophages to produce growth-promoting activity in cell culture. Guinea pig peritoneal macrophages cultured *in vitro* in medium containing PPPS release into the medium, either directly or indirectly, a factor (or factors) that stimulates the proliferation of guinea pig wound fibroblasts. This macrophage-dependent, fibroblast-stimulating activity (MFSA) is nondialyzable, heat stable (56 C for 30 minutes), and requires culture *in vitro* for demonstration of activity. The relationship between MFSA and other growth factor(s) has not yet been determined. In contrast to the macrophage, lymphocytes prepared from mesenteric lymph nodes produced no fibroblast-stimulating activity. (Am J Pathol 84:501–514, 1976)

WE HAVE BEEN STUDYING the *in vitro* growth of diploid guinea pig wound fibroblasts derived from explants to determine which factors are involved in the stimulation of fibroblast proliferation in healing wounds. Recent *in vivo* experiments using guinea pigs, in which a monocytopenia was induced and in which phagocytosis was prevented in healing skin wounds,[1] indicated that the presence of active macrophages in the early stages of repair was an essential prerequisite to subsequent fibroblast proliferation within the wounds. Our observations suggested that, in addition to being the primary cell responsible for wound debridement, macrophages were also required for the production of a factor(s) that promotes fibroblast proliferation. We postulated that such a factor could be produced directly by, or a result of activation of, macrophages or as a result of the breakdown of specific wound components by the macrophages.[1]

In this report, we present evidence for the production by macrophages *in*

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vitro of such a factor(s). This factor(s) stimulates the proliferation of fibroblasts *in vitro,* is nondialyzable, heat stable (56 C for 30 minutes), and is not stored within macrophages, but requires either culture of the macrophages for 6 hours or stimulation of phagocytosis for the production of the proliferation-stimulating activity.

Materials and Methods

Animals

Male Rockefeller guinea pigs (400 to 500 g) were used throughout.

Culture Medium

A modified Dulbecco-Vogt medium[2] based on that originally described by Eagle was used for all the *in vitro* cultures. The relevent sera were added to this basic medium. Medium will be referred to in subsequent sections by the type and concentration of serum within the medium, e.g., 10% fetal calf serum (FCS), 5% blood serum (HBS), or 5% platelet-poor plasma serum (PPPS).

Preparation and Culture of Macrophages

Peritoneal exudate cells were prepared either from unstimulated guinea pig peritoneal cavities, or from peritoneal cavities 4 to 5 days after injection of 10 ml sterile mineral oil (Muri-Lube Heavy, Invenex Ltd.). The peritoneal cavity was washed with 50 ml phosphate-buffered saline (PBS) (containing 10 units heparin/ml). The cells were washed three times with PBS (no heparin) and suspended in 5% PPPS. The macrophages were then plated out in 10-cm Falcon plastic petri dishes in 5% PPPS, incubated for 1 hour at 37 C to allow the macrophages to adhere, and then washed to remove nonadherent cells, resulting in a population of essentially pure macrophages. The medium was replaced with 10 ml fresh 5% PPPS, and the macrophages were incubated at 37 C in a moist incubator gassed with 5% CO_2–95% air. After the relevent incubation period (see below), the medium was removed, centrifuged at 20,000*g* for 30 minutes, and filtered through a 0.22-μ Millipore filter.

Lymphocyte Preparations

Lymphocytes were prepared from guinea pig mesenteric lymph nodes as described previously.[1,3]

Fibroblast Cultures

Fibroblasts were cultured from granulation tissue obtained from guinea pig skin wounds 5 to 7 days after infliction of the wounds. The granulation tissues were dissected free of surrounding skin and cut into small pieces

for explanting. The explants were incubated in 10% FCS, and the cells growing from the primary explants were subcultured by trypsinization. Cells from the second to the fifth subcultures were used to test the effects of macrophage factors on cell proliferation.

Preparation of Blood Serum and Platelet-Poor Plasma Serum

Human blood from young adult males was collected in plastic syringes using sodium citrate (13 μmol/ml) as anticoagulant. The blood was divided into two fractions. The first fraction was allowed to clot by addition of $CaCl_2$ (20 μmol of $CaCl_2$/ml) and was incubated for 2 hours at 37 C to permit the reaction to go to completion. The clot was removed by centrifugation, and the resultant serum was dialyzed against Ringers solution and heat inactivated at 56 C for 30 minutes. The second fraction was spun twice at 250*g* for 10 minutes, and the plasma removed. An aliquot of the platelet-rich plasma was taken for the preparation of purified platelets by the procedure of Tangen *et al.*,[4] and the remainder was centrifuged at 20,000*g* for 30 minutes. The resultant platelet-poor plasma was calcified, allowed to clot in similar fashion to the above, dialyzed against Ringers solution, and heat inactivated at 56 C for 30 minutes.

Assay of Fibroblast Proliferation Stimulating Activity

The assay used in these experiments was based on that described by Ross *et al.*[5] Several replicate 35-mm Falcon plastic petri dishes were prepared, and 3×10^4 fibroblasts/dish were plated out using a Cornwall syringe in medium containing 10% FCS (1.5 ml medium/dish). Eighteen hours after plating the medium was removed, the cells were washed, and the medium replaced with medium containing 1.1% FCS. The cells became quiescent in this serum concentration after 4 to 5 days, and the dishes were then divided into a number of groups to which the relevant medium to be assayed for growth-promoting activity was added. The cells were counted every 2 days by hemocytometer counts after trypsinization. Medium was changed three times weekly.

Previous studies have demonstrated that cells grown in culture in the presence of a pool of 5% serum derived from platelet-free plasma (PPPS) rapidly become quiescent when compared with cells grown in a pool of 5% whole blood serum (HBS) derived from the same pool of blood (see Text-figures 1 and 2). In contrast with other test systems, when cells are quiescent in low serum concentrations (0.5 to 1.0%) and are consequently deprived of nutrients, cells in 5% PPPS have, in theory, all of the plasma constituents available to them with the exception of material released from the platelets during the platelet release reaction. *In vivo*, cells

routinely have plasma constituents available, in contrast to serum components, which would be available only after injury. Thus, we would suggest that cells that are quiescent in culture in the presence of 5% PPPS may be more closely analogous to quiescent cells *in vivo* than cells grown in the presence of low concentration of whole blood serum.[6]

Results

Effects of Medium From Macrophage Cultures on Fibroblast Proliferation

These studies took advantage of the observation that cell-free plasma serum (PPPS) did not stimulate fibroblast proliferation but rather maintained the cells in a state of quiescence in order to study the ability of macrophages to produce stimulatory activity.[5,6] By culturing macrophages in 5% PPPS, any factors produced by the macrophages should be detectable. This also permitted the use of the identical medium for cultivation of both the macrophages and the fibroblasts in the test system.

Macrophages cultured in monolayers in 5% PPPS markedly increased the proliferative acivity of the medium (Text-figures 1 and 2). Whereas in 5% PPPS alone, only slight short-term stimulation of fibroblast proliferation was observed, medium in which macrophages had been cultured stimulated fibroblast proliferation to a greater extent than even 5% HBS. The magnitude of this stimulatory effect was dependent on the number of macrophages and the duration of the cultures. The effects of both unstimulated peritoneal macrophages and macrophages from the mineral oil–stimulated peritoneum were determined. No differences in the observed effects were found for equivalent numbers of macrophages (Text-figure 2).

The effect of macrophage numbers on the stimulation of fibroblast proliferation was determined by assaying medium taken from 6-hour cultures of different numbers of macrophages, ranging from 10^6 to 10^7 macrophages/10 ml culture medium. Optimal activity was found in cultures with approximately 3.6×10^6 macrophages/10 ml 5% PPPS.

Medium from cultures containing 3.6×10^6 macrophages/10 ml 5% PPPS after incubation for varying time periods, between 1 and 24 hours, was assayed for fibroblast-stimulating activity. Optimal activity was found after a 6-hour culture period. Culture for up to 24 hours did not increase the level of activity, whereas shorter periods in culture resulted in correspondingly lower levels of activity.

Replacement of the medium after a 6-hour culture period with fresh 5% PPPS for a further 6-hour culture period resulted in equivalent fibroblast-stimulating activity in the second batch of medium as in the first.

Effects of Modulation of Macrophage Activity on the Production of Fibroblast-Stimulating Activity

The effects of phagocytosis on the production of fibroblast-stimulating activity was determined by addition of latex particles (1.1-μ; Dow Chemical Co.) to the macrophage cultures. Before use, the latex particles were washed twice with PBS and suspended in 5% PPPS at a concentration of 6×10^9 particles/ml; 6×10^8 latex particles (0.1 ml) were added to each dish of macrophages. For a given number of macrophages, addition of latex resulted in a slight increase in fibroblast-stimulating activity (Text-figures 1 and 2). This increase was not sufficient to suggest that phagocytic activity produced the increased levels.

Zymosan particles (Sigma Chemical Co.) were added to macrophage cultures at a concentration of 50 μg/ml to determine the effects of exocytosis on production of fibroblast-stimulating activity.[7] Medium from such cultures did not exhibit increased levels of activity over macrophages cultured in the absence of zymosan.

Killing of macrophages by repeated freezing and thawing in 5% PPPS resulted in less than half the fibroblast-stimulating activity than did culture of the same number of viable macrophages for 6 hours (Text-figure 1).

Effect of Lymphocytes

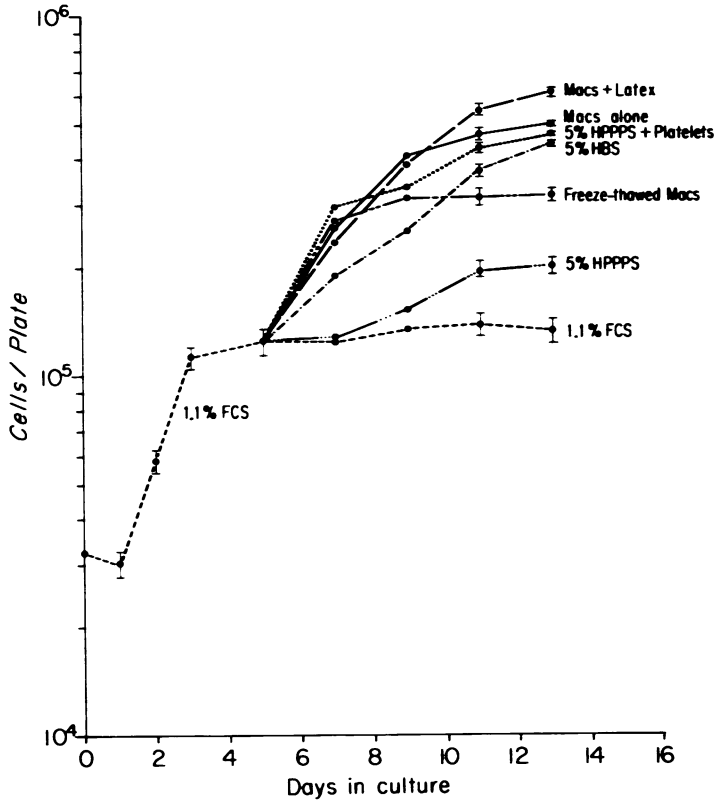
Lymphocytes were cultured in 5% PPPS, in similar numbers and fashion as macrophages, and the resultant medium assayed for fibroblast-stimulating activity. Fibroblast-stimulating activity by lymphocytes was no greater than PPPS alone (Text-figure 2). Addition of latex or zymosan to the lymphocyte cultures did not result in the production of any activity.

Properties of Macrophage-Dependent, Fibroblast-Stimulating Activity

Extensive dialysis of medium from macrophage cultures against medium containing no serum resulted in no loss of macrophage-dependent, fibroblast-stimulating activity (MFSA). Heating at 56 C for 30 minutes also resulted in no loss of activity, and the activity was not destroyed by repeated freezing and thawing.

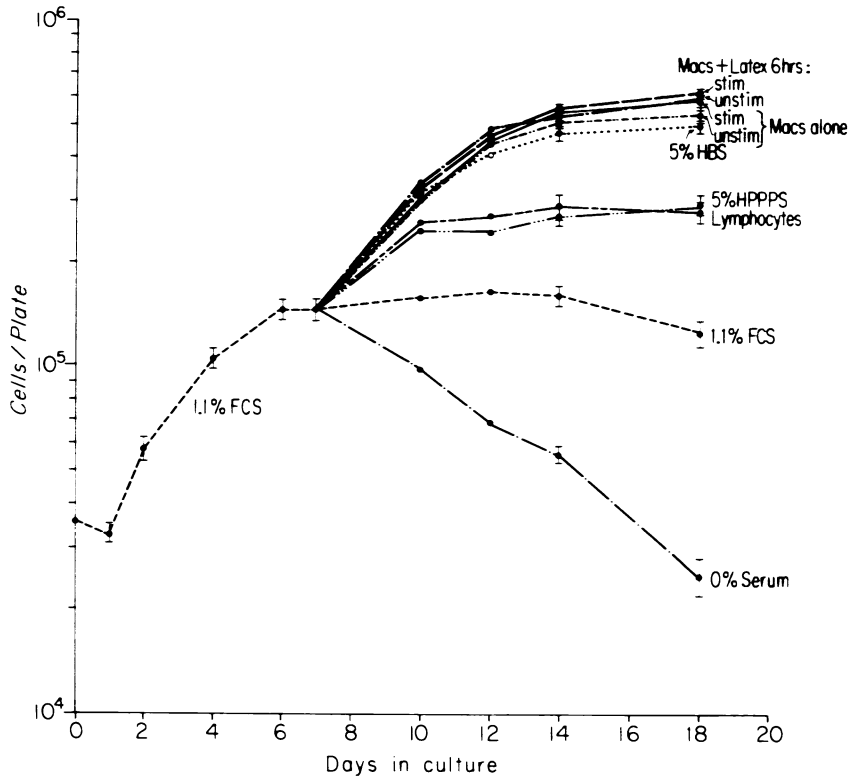
The Platelet Factor

The differences in growth behavior between fibroblasts cultured in 5% HBS and in 5% PPPS also demonstrate the effects of platelet factor(s) on cell proliferation and are shown in Text-figure 1. Whereas in 5% PPPS only slight stimulation of fibroblast proliferation was observed, in 5% HBS the cells grew logarithmically for at least 6 days and reached a much higher cell density before ceasing to proliferate. This difference was



TEXT-FIGURE 1—Response of wound fibroblasts in cell culture to platelet factors and macrophage factors. Equal numbers of guinea pig skin wound fibroblasts were added to a large series of 35-mm petri dishes and incubated in medium containing 10% fetal calf serum for 18 hours. The medium was then changed to 1.1% fetal calf serum (*1.1% FCS*). After 5 days, the dishes were separated into seven groups. One group was incubated with 1.1% FCS. The remaining groups were incubated in medium containing 5% dialyzed serum from pooled human whole blood (*5% HBS*); 5% dialyzed plasma serum from the same pool of human whole blood in which no platelets were present during the process of calcification to form serum (*5% HPPPS*); 5% dialyzed plasma serum which had been exposed to an equivalent number of platelets derived from the same pool of whole blood during the process of serum formation (*5% HPPPS + Platelets*); and three different sets of media that had previously been exposed to macrophages. These three media contained 5% HPPPS, and were identical in each of the three cases. In one case, the macrophages were left in culture for 6 hours (*Macs alone*); in the second case, the macrophages were exposed to latex particles during the 6-hour culture period (*Macs + latex*); in the third case, the macrophages were killed by freezing and thawing several times without a period of culture (*Freeze-thawed Macs*). This experiment demonstrates that platelets provide the factor(s) in whole blood serum necessary for fibroblast proliferation and that macrophages in culture can provide equivalent or greater activity in the absence of the platelet factor(s).

generally a threefold, but ranged up to a tenfold, difference in different experiments. Readdition of purified platelets to platelet-poor plasma prior to clotting reconstituted the activity of the PPPS to levels equivalent to that of HBS (Text-figure 1). Isolation and characterization of the platelet factor is ongoing. The comparison of this factor with that derived from



TEXT-FIGURE 2—Response of wound fibroblasts to media derived from macrophage and lymphocyte cultures. The design of these experiments was the same as for those demonstrated in Text-figure 1 in which 5% dialyzed human plasma serum prepared in the absence of platelets (5% *HPPPS*) served as a base for the growth of macrophages and of fibroblasts in culture. Medium containing 5% HPPPS was exposed to macrophages that were derived from mineral oil–stimulated peritoneal cavities (*stim macs*) or unstimulated peritoneal cavities (*unstim macs*) of guinea pigs, and subsequently either left in culture for 6 hours or exposed to latex particles for the 6-hour culture period. In a similar experiment, lymphocytes derived from mesenteric lymph nodes were cultured under the same conditions. These experiments demonstrate that macrophages derived from both mineral oil–stimulated and unstimulated peritoneal cavities provide stimulatory factors for fibroblast proliferation in equivalent amounts under these circumstances, whereas lymphocytes do not provide any stimulation.

macrophages will be important if we are to understand the role of each of these in fibroblast stimulation.

Discussion

Our earlier studies of the role of the monocyte macrophage in wound repair demonstrated clearly that decrease in the numbers of macrophages in wounds coupled with impairment of phagocytosis of those residual macrophages present in the wounds interfered with the usual sequence of

events leading to fibroblast proliferation and connective tissue formation.[1] Although these studies demonstrated the importance of the monocyte/macrophage in fibroplasia, particularly in terms of wound debridement, they did not provide direct information about the role played by the macrophage vis-à-vis the fibroblast.

Platelets may provide the principal mitogenic activity to serum generally required for growth of cells in culture. However, cells *in vivo* would not be presented with this factor unless there was injury and associated hemorrhage and coagulation. In the case of wound repair, this would only be important during the first 48 to 72 hours. Therefore, it became important to look for another source of mitogenic activity in wounds at the later time intervals. The present observations suggest that the macrophage provides an important component in this role.

The Macrophage Factor

The experiments described in this report clearly demonstrate that guinea pig macrophages cultured *in vitro* release a factor(s) into the culture medium that directly or indirectly stimulates the proliferation of guinea pig wound fibroblasts in culture. This factor will be referred to as *macrophage-dependent, fibroblast-stimulating activity* (MFSA).

We have considered several possible mechanisms for the production of this factor. MFSA could be produced directly, either as a result of synthesis or as a result of activation of a precursor stored within the cells. Alternatively, MFSA could be produced indirectly by activation of an inactive precursor present in the PPPS. This activity could also be produced as a result of degradation by the macrophages of an inhibitor of fibroblast proliferation normally present in the PPPS.

Macrophages from both unstimulated and mineral oil–stimulated guinea pig peritoneal cavities produce equivalent amounts of MFSA. The optimal time period for the production of MFSA was found to be 6 hours. Increasing the duration of culture to 24 hours did not result in greater activity than that found in 6-hour cultures. However, shorter culture periods resulted in correspondingly lower levels of MFSA. Culture of macrophages for 6 hours followed by replacement of the culture medium with fresh medium for a further 6-hour period resulted in equivalent activity in the second batch of medium as in the first.

Killing of macrophages by repeated freezing and thawing without a period of culture resulted in much lower levels of activity than did culture of similar numbers of macrophages in the same medium. Thus, some MFSA may be stored within macrophages, but to a lesser extent than in active macrophages, since culture of macrophages was required for the

demonstration of large amounts of activity. MFSA could be produced either directly, by synthesis by macrophages, or indirectly as a result of synthesis by macrophages of a substance that activates an inactive precursor in PPPS or, conversely, that degrades an inhibitor in PPPS.

Use of agents such as latex to stimulate phagocytic activity by the macrophages indicated that, while MFSA levels were slightly increased, this increase was not sufficient to warrant the assumption that the higher levels were a result of phagocytic activity. Use of zymosan particles, which have been shown to stimulate exocytosis of lysosomal enzymes from macrophages rather than phagocytosis,[7] did not result in levels of MFSA greater than that of macrophages cultured alone. However, the possibility still clearly exists that MFSA may be related to lysosomal enzymes. The ability of proteases to stimulate cell division has received much attention recently but remains a topic of controversy.[8-11]

Macrophages have also been shown recently to produce a dialyzable factor that is inhibitory to cell proliferation in culture. Calderon *et al.*[12] used mouse peritoneal exudate cells and showed that at higher cell numbers than those used in our experiments (30 times our optimal concentration) they inhibited fibroblast proliferation. However, their cells were cultured in medium containing fetal calf serum, and with smaller numbers of macrophages they sometimes observed a stimulatory activity. The relation between their observations and those made in the present report are difficult to explain due to the many differences in the systems and experimental approach.

In contrast to macrophages, lymphocytes prepared from mesenteric lymph nodes produced no fibroblast-stimulating activity, although the cooperative relationship between these two cell types has been previously demonstrated.[13-16]

Other Stimulatory Factors

Multiplication of diploid and nontransformed cells *in vitro* is dependent on certain macromolecular factors present in whole serum.[17,18] A number of different serum factors have been shown to stimulate either cell proliferation or cell migration *in vitro*.[19] These include low density lipoproteins,[20,21] hormones, [22-28] and fractions prepared from whole blood by a number of different procedures.[4,29-32] Conditioned medium factors produced by certain cell strains in culture have also been shown to stimulate cell division.[33,34]

In addition to the above, Chen and Buchanan [32] have recently demonstrated that another factor derived from the clotting sequence, thrombin, is potent in stimulating chick embryo fibroblasts to proliferate in a serum-

free medium. Prothrombin was inactive, but when it was combined with fibroblasts, Factor Xa, and Factor V to produce thrombin, proliferative activity could again be demonstrated. The proteolytic activity of thrombin is well known; however, its role in stimulating chick embryo fibroblasts to proliferate is not clear. Chen and Buchanan have not reported the ability of thrombin to affect other cell types such as 3T3, or diploid mammalian fibroblasts, as reported here. Consequently, it is difficult to relate the significance of their observations with the activity resulting from macrophage activation in the present investigation.

The Platelet Factor

A series of investigations from our laboratory has demonstrated that platelets provide one of the principle components present in serum responsible for smooth muscle cell proliferation in culture.[5,6] While dialyzed serum from clotted whole blood (HBS) promotes proliferation of arterial smooth muscle cells or fibroblasts in culture, dialyzed serum prepared from platelet-free plasma (PPPS) is much less effective, and its potency can be restored by addition of a fraction separately derived from isolated platelets [5,6] (Text-figures 1 and 2). The stimulatory activity provided by platelets has also been confirmed for 3T3 cells by Kohler and Lipton.[35]

It was possible, therefore, to take advantage of the fact that serum derived from platelet-poor plasma lacked the ability present in whole blood serum in order to study the capacity of macrophages to produce proliferative activity, by simply transferring the medium from the macrophages to the fibroblasts to test for production of fibroblast-stimulating activity, after providing the macrophages with appropriate stimuli.

Cells that are quiescent in medium containing 5% PPPS are more likely to be analogous to quiescent cells *in vivo* than cells grown in culture in low serum concentration, which are more analogous to nutrient-deprived cells. Thus, addition of stimulatory factors to PPPS may be more relevant to *in vivo* situations following injury when cells would "see" platelet- or macrophage-derived factors, depending upon the nature of the response to injury.

The fibroblasts used in the present study were derived from explants of 5- to 7-day-old skin wounds, since our previous studies[1,36,37] have indicated that there is a maximal level of fibroblasts in wounds at this stage of repair. In culture, these fibroblasts exhibit the characteristic morphologic and behavioral features typical of fibroblasts, including strict dependence of growth on the presence and amount of serum in the culture medium. The density at which cell proliferation ceases and at which the cells become quiescent increases with increasing concentrations of serum in the culture medium.

Relationship of MFSA to Other Growth Factors

The relationship of MFSA to the growth factors elaborated by thrombocytes[5,6] remains to be elucidated. Both MFSA and the platelet factors are nondialyzable, heat stable (56 C for 30 minutes), and withstand repeated freezing and thawing. However, we do not yet know whether they bear any additional similarities to one another.

Macrophages clearly play an important role in the tissue response to injury. They act as scavengers, phagocytosing foreign and effete cells and tissue debris. They have been shown to produce substances that modulate lymphocyte activity[13-15] and to play an important role in the immune response.[16] Evidence for the production, by silica-treated macrophages, of factors which stimulate the snythesis of collagen by fibroblasts has been presented.[12] However, the potential role of macrophages in stimulating the proliferation of fibroblasts has not been recognized until recently.[1] The local production at sites of injury by focal accumulations of macrophages of factors that stimulate fibroblast proliferation is clearly an important reaction in the response of tissues to injury and in particular in the promotion of the fibrotic response common not only to wound repair but also to many disease entities that entail a substantial inflammatory reaction.

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