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IMMUNOREACTIVE FIBROBLAST GROWTH FACTOR IN CELLS OF PERITONEAL EXUDATE SUGGESTS ITS IDENTITY WITH MACROPHAGE-DERIVED GROWTH FACTOR

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SUMMARY. Peritoneal exudate cells were collected from thioglycollate stimulated mice, extracted an examined for the presence of immunoreactive and bioactive fibroblast growth factor (FGF). The crude extract stimulated in a dose dependent fashion the proliferation of vascular endothelial cells derived from the bovine aortic arch. The extract also showed a parallel and dose-dependent inhibition of binding in a highly specific radioimmunoassay for FGF. The immunoreactive FGF (ir-FGF) contained in the extract was retained on a heparin-sepharose affinity column as is characteristic of pituitary FGF. Reverse-phase HPLC of the macrophage-derived material reveals one biologically active form of FGF which coelutes with the major form of immunoreactivity. The results demonstrate the presence of FGF in these cells and suggest that at least one of the hitherto unidentified mitotic activities in these extracts is due to a mitogen indistinguishable from FGF.

The direct involvement of macrophages in inflammatory diseases, host defense mechanisms and injury repair have led many investigators to establish the existence of several macrophage-derived growth factors (1,2) that may directly affect these processes. One such substance, MDGF, never characterized so far, is a potent mitogen for nonlymphoid mesenchymal cells including fibroblasts, smooth muscle and endothelial cells (3,4). Although similar activities have been reported for brain and pituitary fibroblast growth factor (FGF) (5,6) the inability, until recently, to ascribe a structure for any of these mitogens has precluded any analysis of similarity. The recent isolation and partial structural characterization of bovine pituitary FGF (7,8) has enabled us to examine the possibility that the several factors endowed with these mitogenic activities could be structurally related if not identical to FGF. Using

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sequence specific antibodies, we demonstrate that an immunoreactive fibroblast growth factor is present in extracts of peritoneal exudate cells that are comprised mostly (75%) of macrophages (4). The implication of this finding in understanding the etiology of macrophage-induced neovascularization (4,9), injury repair and wound healing (3,10) is discussed.

MATERIALS AND METHODS

Preparation and Extraction of Tissues:

Thioglycollate-stimulated peritoneal macrophages were prepared by the method of Gallily et al (11) as described by Shiigi (12). Four days after thioglycollate stimulation, the peritoneal cells were harvested by intraperitoneal lavage with Hanks' Basal Salt Solution and washed extensively by centrifugation. The yield of cells was estimated at 1-5 X 10^7 cells/mouse with the total 250 mg of tissue (wet weight) obtained from the 9 treated mice.

The cell pellet was extracted at 4°C in 2.5 ml of 0.15 M $(NH_4)_2SO_4$, pH = 4.5 by sonication for 60 seconds. The extract was stirred for 2 hours and centrifuged at 12,000 rpm in an Eppendorf centrifuge. The supernatant was neutralized and aliquots removed for bioassay and radioimmunoassay. The remaining material was processed by heparin-sepharose affinity column and reverse phase HPLC for the purification of fibroblast growth factor as described (8).

Radioimmunoassay (RIA) for FGF:

Antibodies generated against a synthetic replicate of the N-terminal of FGF were used to develop the RIA for FGF (7,13). The antisera recognize the synthetic hapten and native FGF on an equimolar basis and are highly specific for the amino-terminal of FGF. They also recognize bovine, human, ovine, murine and mouse FGF with no indications of species specificity (manuscript in preparation).

Bioassay for FGF:

Mitogenic activity was assessed by the method of Gospodarowicz et al (8) using cloned vascular endothelial cells derived from the bovine aortic arch. Cells were plated at an initial density of 2-3 X 10^4 cells/ml in 24 miniwell dishes. Eight hours later, 10 µl aliquots of the test samples were added to the cells. The treatment was repeated 48 hours later without changing the medium and the number of cells was determined using a Coulter Counter on the 5th day of the experiment.

Purification of Macrophage-Derived FGF:

Heparin-sepharose was obtained from Pharmacia and prepared according to the specifications of the manufacturer. Columns built with this gel (0.5 ml bed volume) were loaded with the extract and eluted by gravity. The column was then washed with 2 ml of successive passes of 0.15, 0.6, 1.1, 2 and 3 M NaCl in Tris-Cl buffer, pH = 7.4. Aliquots of each fraction were removed for RIA and the 2 M and 3 M eluates were pooled and further processed by HPLC by loading directly onto a C4 µbondapak column (7,8) and eluting with a gradient of 26-43% acetonitrile in 0.1% trifluoroacetic acid. Aliquots of column fractions were removed, dried in vacuo and tested for the presence of bioactive and immunoreactive FGF as described above.

RESULTS

As shown in table 1, macrophage extracts have the capacity to stimulate the proliferation of vascular endothelial cells *in vitro*. There was a three fold increase in the cell number with 2 µl of the extract that was not different from that obtained with an FGF standard (1 ng/ml). The RIA for FGF shows that these extracts give a parallel and dose dependent crossreactivity with the antibodies raised against a synthetic replicate of the amino terminal of FGF (Figure 1). Quantitation by the RIA estimates the extract to contain 20 pmol/ml of ir-FGF. This amount of ir-FGF is consistent with the potency of the extract to stimulate the proliferation of bovine vascular endothelial cells *in vitro*.

The elution profile of the extract after heparin-sepharose affinity column is shown in figure 2. Although some ir-FGF was found to elute early with .6 M NaCl, the majority of the ir-FGF retained by the column was eluted with 2 M and 3 M NaCl. This observation is consistent with the high affinity of bovine pituitary and brain FGF (8) for heparin-sepharose and is similar to that reported by Shing and his colleagues (14) for chondrosarcoma-derived growth factor (CDGF) and Maciaz et al (15) for endothelial cell growth factor (ECGF).

Reverse phase HPLC of the pooled 2 M and 3 M eluates from the heparin sepharose affinity column is shown in fig. 3. The RIA for FGF in aliquots of the column fractions revealed two forms of immunoreactivity. One, quantitatively minor in terms of ir-FGF content had no detectable biological activity,

TABLE 1: MITOGENIC ACTIVITY OF PERITONEAL EXUDATE CELL EXTRACT

Amount of Extract (µl)	Cells/dish (X 10^{-4})
0	4.0 ± 0.2
0.02	7.2 ± 0.3
0.2	10.4 ± 1.1
2	12.5 ± 0.7
FGF (1 ng/ml)	12.3 ± 1.1

Aliquots of the extract (0-2 µl) were tested for their ability to stimulate the proliferation of bovine aortic arch endothelial cells *in vitro*.

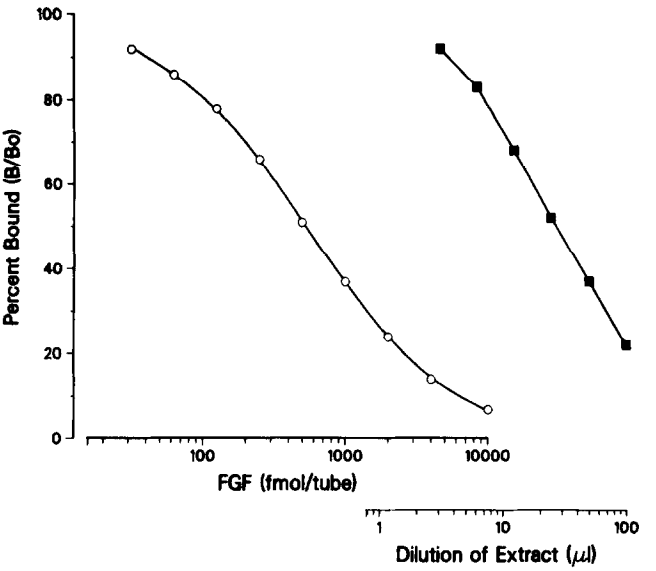

Figure 1. Crossreactivity of Macrophage-Derived FGF. Aliquots of the neutralized extract were serially diluted and assessed for their immunoreactive FGF (ir-FGF) content by RIA.

was not retained by the column and was detected in the wash through. The second form of ir-FGF eluted with a retention behavior similar to that of native pituitary or brain FGF (7,8) and possessed all of the mitogenic activity detected in the column effluent.

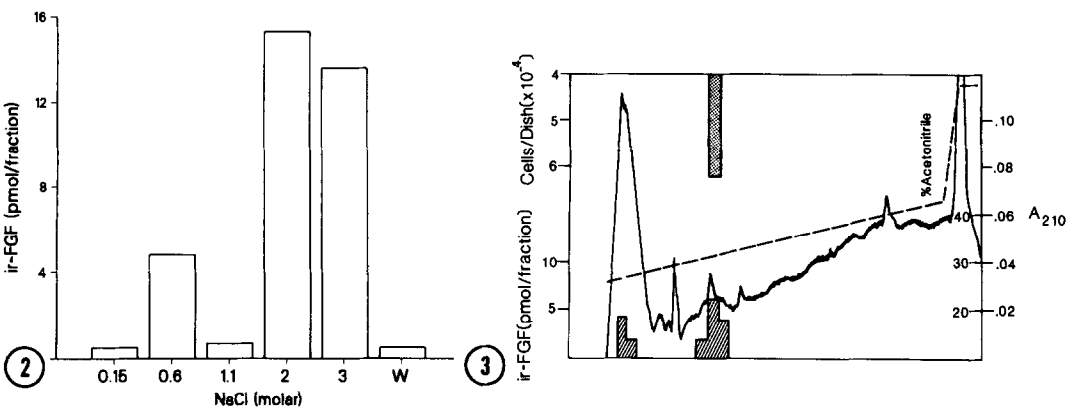

Figure 2. Heparin-Sepharose Affinity Column Chromatography of the Peritoneal Exudate Cell Extract. Aliquots of each fraction were assessed for ir-FGF content by RIA.

Figure 3. Reverse-Phase HPLC of Macrophage-Derived FGF. The pooled 2 M and 3 M NaCl eluates of the Heparin column were eluted with a gradient of 26-43% acetonitrile in 0.1% TFA. Aliquots of the column fractions were tested for their ir-FGF content and ability to stimulate the proliferation of bovine aortic arch endothelial cells in vitro.

DISCUSSION

We have demonstrated that extracts of peritoneal exudate cells contain a biological and immunoreactive FGF-like mitogen. From our previous studies (7,8,13) it appears indistinguishable from bovine brain or pituitary FGF. It is a potent stimulator of vascular endothelial cells *in vitro*; it crossreacts with sequence specific antisera to bovine FGF; it is retained on heparin sepharose affinity columns and it has a similar retention behavior on HPLC as native pituitary or brain FGF.

Although the specific cell type in the peritoneal exudate containing the ir-FGF is not known, the cell preparation used in these experiments is known to produce 75% activated macrophages (4). Macrophages secrete a wide variety of growth promoting factors which play important functions in the host defense system. These include such diverse activities as classic interferon, lymphocyte activating factor, endogenous pyrogen, acute phase protein synthesis factor, angiogenesis factor, fibroblast and endothelial cell growth factor and others (3). Because the biochemical nature of these molecules is not known, however, some of the ten or more mitogens detected in macrophage extracts may turn out to be the same substance. This is what appears to be the case for macrophage-derived growth factor (MDGF) and fibroblast growth factor (FGF). Both molecules have angiogenic activity, can induce neovascularization, are associated with tissue repair and are mitogenic for cells derived from the primary and secondary mesenchyme (1-6,16). From the results presented here they are structurally related if not identical. Isolation of a large enough quantity of the macrophage derived FGF-like mitogen for structural analysis should answer this question in the near future.

The detection and demonstration that fibroblast growth factor is present in extracts of macrophages has widespread implications as to its possible function *in vivo*. Gospodarowicz (5,16) demonstrated that pituitary FGF could induce such processes as angiogenesis, limb regeneration and injury repair. In view of the results reported here, such physiological activities in the normal animal may not be mediated by circulating pituitary FGF per se but may well be

due to macrophage-derived FGF locally released. Therefore the infiltration of macrophages at sites of injury and inflammation make macrophage-derived FGF a likely candidate to mediate the paracrine tissue healing process so far attributed primarily to a specific macrophage-derived growth factor. This particular FGF-mediated macrophage function may prove the underlying mechanism that mediates several pathophysiological manifestations of mesodermal cell growth including the formation of atherosclerotic plaque (10), proliferative glomerulonephritis (7,17) and disorders of smooth muscle growth (18).

These results also raise questions as to the origin of FGF from pituitary and other tissues (manuscript in preparation). Macrophages, as members of the mononuclear phagocyte system, circulate in blood as monocytes and differentiate into macrophages in tissues such as the liver, lung and peritoneum. They remain in tissues for periods of up to several months as quiescent, resident cells which may become inflammatory or activated after stimulation. It will be important, in view of the results presented here, to determine the exact role FGF plays in macrophage-mediated events of the immune response and to determine the source and nature of the FGF secreting cell in non-hematopoietic cell systems.

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