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STUDIES ON MACROPHAGE-MEDIATED COMPENSATORY GROWTH AND ON EMBRYONIC GROWTH: DEVELOPMENT OF A mRNA PHENOTYPING METHOD

by

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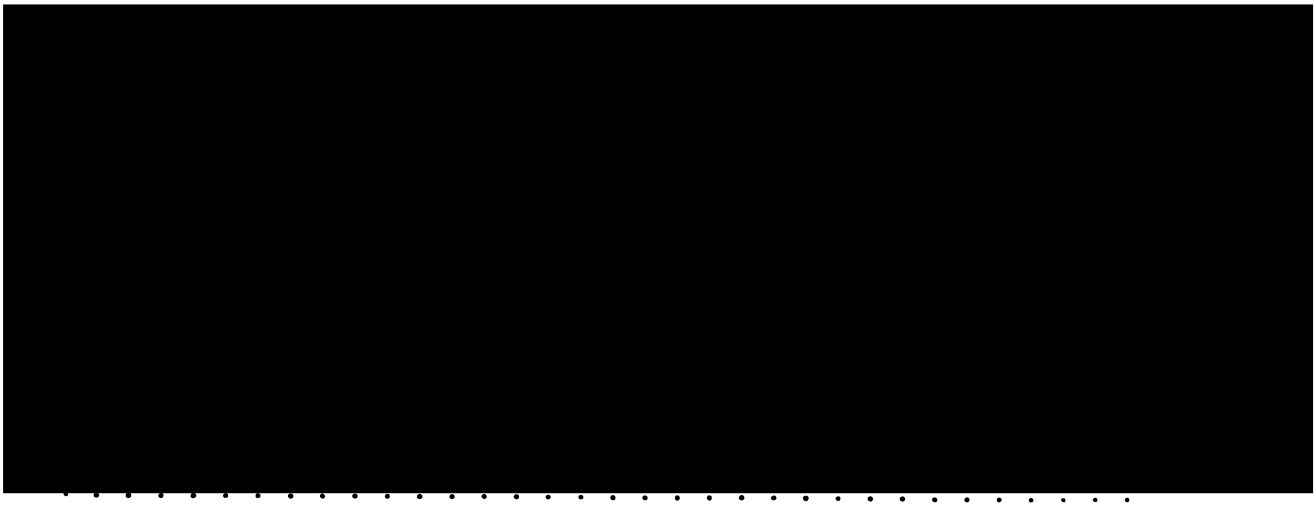

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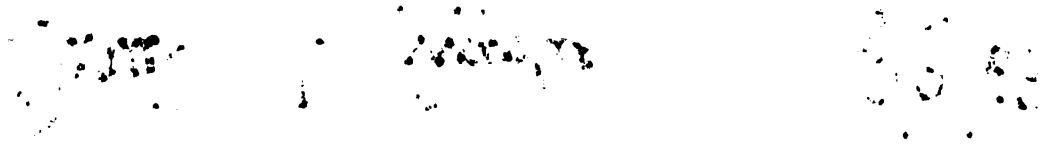

Studies on macrophage-mediated compensatory growth and on embryonic growth: development of a mRNA phenotyping method

Daniel Allen Rappolee

Abstract

Growth factors are important in macrophage controlled wound healing, nerve regeneration, anemia-induced erythropoiesis, platelet controlled hemostasis and inflammation, and in mammalian preimplantation embryonic growth.

We have developed a technique called "single-cell mRNA phenotyping" which has the characteristics of extreme sensitivity (as few as 10 copies of mRNA), rapid qualitative screening for multiple mRNA transcripts, and a resolution of less than 3-fold differences in RNA input. The technique consists of linked methods for purifying single cell RNA, reverse transcription primed by oligo dT or sequence specific primers, and sequence-specific primed polymerase chain reaction. We have used the technique to mRNA phenotype cells participating in compensatory and embryonic growth.

a) Macrophage growth factor expression in wound healing.

Ablation of macrophages slows the regeneration of cells required for wound healing. The cell types which divide during wound healing in vivo are stimulated to divide by various growth factors in vitro. We tested the hypothesis that wound macrophages express these growth factors in vivo by isolating wound macrophages from sub-epidermal cylinders in mice and phenotyping their growth factor mRNA. We found that wound macrophages express TGF-β, PDGF A, IL-1 and two growth factors not previously characterized in macrophages, TGF-α and IGF-1. We validated the translation of TGF-α by immunofluorescence studies of wound macrophages. We also found that macrophages express authentic 6 kDa biosynthetically immunoprecipitable TGF-α and 4.5 kb mRNA when challenged in vitro with wound stimuli. Using differential affinity for heparin and a bioassay for mitogenesis, we demonstrated 3 major peaks of mitogenic activity produced by stimulated macrophages (TGF-α, PDGF, and bFGF). Heparin non-binding TGF-α constitutes a major part of the mitogenic activity

produced by macrophages. Taken together with studies demonstrating the importance of TGF-α in angiogenesis, epidermal regrowth, and granulation tissue generation, we infer that TGF-α is a central macrophage-derived growth factor in wound healing.

b) Other models of macrophage- and platelet- mediated compensatory growth.

We have found that macrophages express growth factors which influence nerve regeneration that are expressed in regenerating nerve, and that stimulated macrophage liberate EGF (see Chapter 4 for detail). We have also found that megakaryocytes express TGF-α (but not EGF), PDGF, but not fibrinogen or albumin. Because these polypeptides are all found in platelet α granules we infer that the fibrinogen and albumin are taken up from plasma (see Appendix B).

c) Preimplantation mouse embryo growth factor expression.

Preimplantation mouse embryos can grow in vitro without exogenous growth factors. We tested the hypothesis that these embryos might express endogenous growth factors by mRNA phenotyping. We found that preimplantation mouse embryos express TGF-α, TGF-β, and PDGF A transcripts, but not FGF, EGF, NGF-β or G-CSF. We also found TGF-α and PDGF by immunofluorescence microscopy. We found the expression of a subset of growth factors was temporally specific and speculate that growth factor expression is functionally relevant. We are continuing this study by further characterizing spatial and temporal patterns of growth factor ligand and receptor expression. Using this information we hope to design experiments to test for function of growth factors in early mammalian embryogenesis.

Submitted papers or papers in press reported in this work:

Daniel A. Rappolee and Zena Werb, Macrophage Secretion: A functional perspective, Bulletin of the Pasteur Institute, *in press* 1988.

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Daniel A. Rappolee, Prem Handagama, Zena Werb and Dorothy F. Bainton, Differential expression of growth factor and plasma protein genes in megakaryocytes, manuscript in preparation, 1988.

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Chapter 1

Macrophage Secretion: A Functional Perspective

Introduction

The macrophage is a cell central to both the defense of the organism and homeostasis. Since the time of Metchnikoff, at the turn of the century, it has been studied as a model of endocytosis and cytotoxicity. In the last fifteen years macrophage studies have been expanded to include its pleiotropic differentiation states and labile phenotype. In its many forms the macrophage is found in nearly all tissues and organs. It is a sensitive sentry responding to myriad signals in its milieu, via at least 60 receptors. Its secretory response is selected from an even more varied repertoire of molecules. The more than 100 secreted molecules of the macrophage that constitute this repertoire (Tables 1-5) are the subject of this review.

Macrophages arise from stem cell precursors in the bone marrow, circulate as monocytes in the blood, and migrate into tissues to assume their mature functions. The pleiotropic macrophage has a distinct differentiation state in each tissue: the fixed macrophages of liver (Kupffer cells), bone (osteoclast), bone marrow, epidermis (Langerhans cells), central nervous system (microglia), connective tissue (synovial type A cells), connective tissue (histiocytes), spleen, and the free living macrophages of alveolar spaces in serosal cavities (peritoneal and pleural macrophages) (4,8,12,250). These cells have diverse functions and cellular metabolism. For example, peritoneal macrophages, but not blood monocytes, demonstrate a Pasteur effect (depression of glycolysis by oxygen). However, peritoneal macrophages and monocytes exhibit a Crabtree effect (depression of respiration by glucose), but alveolar macrophages do not (45,107).

The functional state of the macrophage is determined not only by its tissue of maturation but by acute pathological conditions in the milieu. Macrophages may exist in tissues for periods of up to several months as quiescent *resident* macrophages. These cells secrete few neutral proteinases or reactive oxygen intermediates. However, foreign material signals quiescent macrophages to enhanced secretory and/or killing activity. This requires a process

Table 1 ENZYMES AND ENZYME INHIBITORS SECRETED BY MACROPHAGES

Protein	Reference
Plasminogen activator (urokinase)	31, 40, 47, 134
Collagenase I	242,243,249
Collagenase IV (68 kDa gelatinase)	48, 78
Collagenase V (92 kDa gelatinase)	140, 141, 239
Stromelysin	190
Elastase (metallo)	15, 16, 36, 102, 255
Elastase (PMN-type)	75, 186
Cytolytic proteinase	3
Complement: classic pathway, Clq,2,3,4,5,6,7,8,9	6, 23, 69, 127, 176, 202, 222, 263
Complement: alternate pathway, factor B,D, properidin, factor H, I	169, 222, 226
Coagulation factors	171, 172
Lysosomal acid hydrolases (40)	205, 233
Cathepsin L	181
Lysozyme	44, 92
Arginase	119
Angiotensin converting enzyme	212
Lipoprotein lipase	22, 115, 139
α_2-Macroglobulin	105

Table 2 GROWTH AND CELL FUNCTION REGULATORY FACTORS SECRETED BY MACROPHAGES

Factor	References
Interleukin-1	10, 11, 32, 54, 136, 152
Platelet-derived growth factor (c-*cis*,A)	144, 189, 211
Fibroblast growth factor	1, 2, 13, 189
Tumor necrosis factor-α	27, 28, 131, 166
Nonmitogenic angiogenic factor	14, 116
Transforming growth factor-β	7, 189
Erythropoietin	173, 175, 193
GM-CSF	42, 88, 148, 230
Plasmacytoma growth factor/IL6/ interferon-β2	85, 117, 164, 231, 237
Interferon-α	71, 137, 221
Arginase	119
ACTH	215
Bombesin	259
Vitamin D3	96
Nitrates	103, 223
Interleukin-1 inhibitor	
Macrophage-derived growth factor	30, 63, 87, 109, 221, 258

Table 3 CYTOSTATIC AND CYTOCIDAL MOLECULES SECRETED BY MACROPHAGES

Factor	References
Interferon-α	71, 137, 221
Interferon-β_2	85, 117, 232, 237, 164
Tumor necrosis factor-α	28, 236
Complement C1, C2, C3, C4 & C5	23
Complement factor B, D	23, 169, 222, 226
Apolipoprotein E	20, 253
PGE_1, PGE_2	80, 120, 121
Nitrites, nitrates	223, 103
Arginase	119
Cytolytic neutral proteinase	3
Reactive oxygen intermediates ($O^{\bullet}_2$, O^1_2, H_2O,OH_2)	161
Lysozyme	44, 92
Lysosomal hydrolases	233
Vitamin D	96
Interleukin-1	168
TGF-β	7, 189
Purine metabolites	39
Thymidine	219

Table 4 FUNCTIONAL EFFECTS AND REGULATION OF INTERLEUKIN 1

Substances stimulating IL-1 production	Producer cell types	Effects of IL-1
Interleukin-1	Macrophages	*T cell:*
TGF-β	Endothelial cells	
TNF-α	Kupffer cells	Chemoattractant
LPS	Fibroblasts	Comitogen
LTC_4	Keratinocytes	IL-2 receptor induction
C5a	Mesangial cells	
LTD_4	Dendritic cells	*B cell:*
Muramyl dipeptide	Thymic epithelial cells	Chemoattractant
Silica	Microglial cells	Comitogen
Zymosan		MHC class II inducer
Interferons α and β		Maturation inducer
Interferon-γ		
Adherence		*NK cell:*
		Cytotoxicity inducer
		IL-2 receptor inducer
		Macrophage:
		Cytotoxicity inducer
		Reactive oxygen inducer

IL-1 RNA and polypeptide inducer

TNF-α inducer

Thromboxane B_2 inducer

Endothelial cells:

Procoagulant activity inducer

Anticoagulant activity suppressor

Plasminogen activator inducer

Plasminogen activator inhibitor inducer

PAF, PGF_2, PGI_2 inducer

IL-1 inducer

GM-CSF, G-CSF, M-CSF inducer

Adherence of T and B cells,

PMN, macrophages

Tumor cells:

Cytotoxicity

Fibroblast and synovial cells:

PGE_2 inducer

Collagenase inducer

Interferon β_1/β_2 inducer

Proteinase inhibitor inducer

Hyaluronate inducer

GM-CSF inducer

IL-1 inducer

MHC class II inducer

PMN:

Precursor inhibition

Degranulation

Reactive oxygen inducer

Thromboxane β_2 inducer

ADCC inducer

Phagocytosis inducer

Induces adhesion to endothelial cells

C3bi inducer

Induces release from bone marrow

Bone:

Resorption

Cartilage:

Proteoglycan synthesis suppressor

Metalloproteinase inducer

Basophils:

Histamine release

Systemic changes:

Acute phase response (C3,haptoglobin, fibrinogen) increase, Iron decrease (due to PMN lactoferrin secretion)

Fever (reset hypothalamic set point)

Shock

Glucocorticoid induction

References: 4, 11, 19, 25, 31, 32, 38, 45, 50, 54, 59, 60, 65, 76, 81, 86, 123, 124, 133, 135, 150, 151, 156, 167, 168, 170, 177, 179, 182, 183, 189, 195, 202, 203, 206, 214, 224

Modified after Nathan (158). TGF-β (transforming growth factor-beta), TNF-α (tumor necrosis factor-alpha), LPS (lipopolysaccharide), LTβ_4 (leukotriene β_4), LTD_4 (leukotriene D_4), C5a (complement component 5-activated fragments), PAF (platelet activating factor), PGF_2 (prostaglandin F_2), $PG1_2$ inducer (prostaglandin I_2-inducer), GM-CSF (granulocyte-monocyte colony stimulating factor), G-CSF (granulocyte colony stimulating factor), M-CSF (macrophage colony stimulating factor), ADCC inducer (antibody dependent cell-mediated cytotoxicity), C3bi (complement component 3b inhibitor inducer).

Table 5 CLONED MACROPHAGE SECRETORY PRODUCTS

Product	References*
α_1Proteinase inhibitor	
Apolipoprotein E	
Arginase	
C1 inhibitor	
Cathepsin B	
Cathepsin D	
Cathepsin G	199
Cathepsin L	181
Collagenase	73
Complement factor B	
Complement factor 2	
Complement 3	
Complement 4	
Complement 5	
Corticotropin	
Elastase (PMN type)	213
Fibronectin	
GM-CSF	
IFN-β2	

IL-1α	136, 143
IL-1β	95, 143
Lysozyme	44
Alpha$_2$-macroglobulin	
Plasminogen Activator	
Plasminogen Activator	
Plasminogen Activator Inhibitor	
PDGF A	
PDGF B	188
Secretory Leuk. Protease	
Thrombospondin	
TNF-α	
TNF	
TIMP	61
TGF-β	
TGF-α	55
C9	
γ-Fibrinogen	
IGF-I	
Erythropoietin	
Bombesin	
FGF basic	1, 2, 68
C1s	

C1r

Stromelysin

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*Except where noted the references for the cDNA clones are from a comprehensive list of human sequences from a supplement of Nucleic Acids Research (204).

comprising two steps (4,46,91,114,158,162,165,178,216,233,257). Quiescent resident macrophages are induced by microbial products to become *inflammatory* macrophages, which have increased neutral proteinase secretion but low secretion of reactive oxygen intermediates (and therefore low killing capability). This may occur in circumstances, such as blunt trauma or allergy, in which the integrity of the epithelia remains intact. Experimentally, 'elicited' or 'stimulated' macrophages are produced by sterile foreign stimuli and are synonymous with inflammatory macrophages. If epithelial integrity is lost and viable microbes enter, or when immunological cytokines such as γ-interferon (γ-IFN) are produced, resident or inflammatory macrophages progress into the *activated* state. Macrophage subpopulations such as fixed tissue macrophages of liver and bone marrow are incapable of being activated by γ-IFN to produce reactive oxygen intermediates (250). However, macrophages in the activated state have reduced secretion of neutral proteinases and enhanced secretion of reactive oxygen intermediates. Activated macrophages are microbicidal for intracellular organisms or tumoricidal. It is important to realize that the signals that stimulate or activate resident macrophages arise under circumstances that elicit blood-borne monocytes. In addition to resident macrophages monocytes make a large contribution to any population of inflammatory or activated macrophages.

To summarize, the functional state of a population of macrophages is selected from a restricted segment of the repertoire of all macrophages. The selection is determined by signals in the milieu, and by the differentiation state and history of the macrophage population. This review will attempt to correlate macrophage functions with secretory phenotypes. Emphasis will be placed on the most highly characterized secretory molecules, in systems in which in vitro studies of secretion resemble most closely in vivo functional studies. These functional groups constitute the main pathophysiological roles of macrophages. The tables list the macrophage products by functional groupings. Table 5 also lists the rapidly

increasing group of macrophage products for which molecular cDNA clones exist.

The macrophage is a complex and multifunctional cell. At present many of the secretory products are known as 'activities' rather than as molecules. Many activities may result from a single secretory product; e.g., as interleukin-1 was originally characterized as five disparate activities (79). Other secreted 'activities' have been derivatized regurgitated molecules. Such is the case for a suppressor of B cell antibody and DNA synthesis secreted by T cells and modified and resecreted by macrophages (9). The challenge of understanding macrophage secretion is in dissecting the quantity and action of each secretory product and attributing its capability to the functions of the macrophage.

Hemostasis

The role of the macrophage in hemostasis is not well understood. Hemostatsis is maintained primarily by platelets. The rupture of a blood vessel leads to vasoconstriction, which is mediated neuronally and by platelet activation, and platelet plug formation. These two events occur in seconds to minutes. A third event, clotting, also begins in seconds, but is not effectively completed for minutes to tens of minutes. The vessel is returned to hemostasis by clot retraction within one hour of rupture (191). Clot growth is limited by the short half-lives of the procoagulants, by inhibitors of clotting factors, and by fibrinolytic enzymes (261).

Macrophage secretory products may contribute to the control and maintenance of the clot size although macrophages do not contribute much to the mass of the clot. Fibrinolytic clot dissolution is mediated by macrophages, the predominant leukocyte in the inflammatory lesion at 18 hours after vessel rupture, when dissolution takes place. This will be discussed in detail in the section on debridement and fibrinolysis.

Two major secretory products of macrophages, tumor necrosis factor-α and IL-1, may have hemostasis function. Monocytes stimulated by a variety of agents secrete tumor necrosis

factor-α (TNF-α) (32,75,82,150-152) and interleukin-1 (IL-1) (166,167,177,195). Both of these polypeptides have been intensively studied, cloned, and sequenced in recent years (10,11,95,143). TNF-α and IL-1 act upon endothelial cells both to induce procoagulant activity (24,38,163,202) and to reduce anticoagulant activity and to regulate vascular permeability and leukocyte movement into tissue (28). Because the induced secretion and activity of TNF-α and IL-1 from macrophages in vitro peak at 4 hours, the kinetics of these effects on endothelial cells and on inflammation suggest that these polypeptides regulate later maintenance of clot. TNF-α and IL-1 may also contribute to pathologic conditions such as hemorrhagic necrosis, thrombosis, and intravascular coagulation.

Hemostasis is also maintained by intrinsic (humoral) and extrinsic (cellular) coagulation pathways. The extrinsic pathway of coagulation (261) has two factors that distinguish it from the intrinsic/common pathway: VII and III (tissue thromboplastin). Macrophages produce thromboplastin activity (171) and factor VII (143) upon stimulation by the lipopolysaccharide moiety of gram-positive bacterial cell walls. The relative participation of coagulation proteins of macrophages in nucleating the coagulation in platelet clots, or in other processes such as the walling off process of inflammation or of scaffolding in wound healing (discussed below) is unclear.

The pleiotropic nature of the effector molecules secreted by macrophages, as well as the multiplicity of sources for some of these secreted products, makes it more difficult to determine the importance of their contribution to various functions. For example, macrophages secrete platelet-derived growth factor (PDGF) upon stimulation (144,211). This growth factor certainly contributes to atherogenic and wound healing processes (83,84,196). However, it is also a powerful effector of vascular spasm mediated by smooth muscle cells (24). Platelet degranulation delivers the early mass of PDGF; however, macrophages, endothelial cells, and smooth muscle cells all secrete this factor (196), and it contributes to the

vessel tonicity after early spasms.

Nonpolypeptide macrophage secretions such as platelet-activating factor--a phosphoglyceride that causes platelet aggregation and degranulation (101,147)--and prostacyclin--a prostaglandin that inhibits platelet aggregation (220)--are also pleiotropic and of multiple sources.

The fibrinolytic activity of macrophages has been intensively studied and is relatively well understood. This activity is important in the debridement of extravascular clotting and will be discussed in the section on debridement and fibrinolysis. Hemostatic clots dissolve in a few hours to days after formation. Many stimuli induce macrophages to secrete plasminogen activator, which is then bound to a membrane receptor (40,41,106,134,198,215,227,235,240,241,248,262). Macrophages adhere to the matrix molecules of the fibrin clot, such as fibrin and fibronectin, and locally form pockets protected from overwhelming concentrations of plasma proteinase inhibitors, which would defeat fibrinolytic proteinases (89,90). Macrophages also secrete proteinase inhibitors, including α_1-proteinase (α_1PI) inhibitor, plasminogen activator inhibitor-2 (PAI-2), and α_2-macroglobulin (α_2M) and may limit the proteolysis they initiate (18,41,105,154,238,240,246,247).

In summary, macrophage participation in hemostasis is poorly understood. Stimulated macrophages secrete several products capable of participating in early as well as late phases of hemostasis. The kinetics of macrophage appearance and stimulation suggest that macrophages participate in late hemostatic function. Macrophages are strongly involved in clot dissolution.

Inflammation

Tissue trauma leads to histamine release, and there is an ensuing increase in vascular

permeability and edema. The combination of leaking fibrinogen and tissue exudates leads to extravascular coagulation. This 'brawny edema' slows the spread of invading microbes. Inflammatory factors liberate pools of mature bone marrow neutrophils into blood. Factors such as leukotrienes, PDGF, bacterial proteins, and complement fragments attract neutrophils and monocytes to inflammatory foci. After about 12 hours macrophages, which, unlike polymorphonuclear leukocytes (PMN), are not stored in bone marrow, become the primary cell in inflammatory foci (193).

CSa, TNF-α, IL-1, LTB_4, transforming growth factor-β (TGF-β), and PDGF are either chemoattractive to monocytes or induce monocyte-endothelial cell adherence (147,218,244). Macrophages predominate in these lesions for several reasons. Macrophages survive the increasingly acid environment of the focus better than PMN, are more phagocytic than PMN, and synthesize and secrete lysosomal acid hydrolases with optimal activities at acid pH. Macrophages are well suited to the later inflammatory phases of killing, pus formation, and clearing of inflammatory foci. These events precede the resolution of acute inflammation. Persistent injurious agents (such as tubercle bacilli or silica) that resist macrophage disposal lead to chronic inflammation. In this case macrophages aggregate and fuse to form long-lived granuloma giant cells, which have many of the properties of inflammatory macrophages.

The four cardinal signs of inflammation are contributed to by several lipid derivatives secreted by stimulated macrophages: prostaglandin E (PGE), leukotriene C and D (LTC and LTD), and thromboxane A_2. PGE_1 and PGE_2 cause pain, vascular dilation leading to edema, and erythema (100,200,220). LTC_4 (also known as the slow-reacting substance of anaphylaxis, SRS-A) and LTB_4 mimic histamine by increasing vascular permeability in postcapillary venules. LTC_4 also causes terminal arteriole constriction, but acts at concentrations three to four orders of magnitude less than histamine (100).

Stimulated macrophages secrete proteinases important in acute inflammation.

Urokinase-type plasminogen activator (uPA) initiates fibrin degradation and it initiates pathways that produce bradykinin (causing pain), and complement fragments and fibrin split products, which attract more leukocytes (62,261). Macrophage uPA also aids macrophages in their penetration of extravascular clots and entrance into tissue. Macrophages produce angiotensin converting enzyme, which cleaves angiotensin I to vasoconstrictive angiotensin II (212). This enzyme is exceptional compared to macrophage neutral proteinases in that glucocorticoids do not attenuate its production (228). Several other neutral proteinases, collagenases, stromelysin and elastase, are secreted by inflammatory macrophages, perhaps allowing the cells to move through the interstitium. These proteinases are also important in wound debridement and remodeling and are discussed below.

Resident, inflammatory, and activated macrophages all produce lysozyme, an enzyme with bacterial cell wall muramic acid polymer as substrate (44,92), and the expression of this enzyme is markedly increased with macrophage activation (S. Gordon personal communication).

Yeast and bacterial cell walls stimulate secretion of several complement components in inflammatory macrophages. Complement component C3 secretion is the most important of these, because it amplifies the effects of the early antibody-activated factors, C1qrs and C2, and generates split products with the chemotactic and opsonic functions for macrophages and neutrophils (229). C3 is also a major plasma protein, but a 10-fold excess of an inhibitor of active C3 in plasma makes local production of macrophage C3 important. Macrophages also produce the components of both the alternate and classical pathways involved in early recognition and amplification phases. Complement products are of primary importance in killing and clearing inflammatory foci. Macrophages control the extent of the reaction in a negative direction as well by secreting several complement inhibitors (41,105,162,228). Some components have short half-lives as well. Finally, complement synthesis slows after

stimulating opsonized microbes and immune complexes are removed and are negatively regulated by α_2-M-proteinase complexes (113) binding to macrophage receptors.

Later, in the pus-forming stages of inflammation, neutral proteinases are inactivated in the acid environment are created, in part, by lysosomal degranulation. As much as 25% of the total lysosomal content of macrophages can be released during phagocytosis or when organisms resist phagocytosis (205,229,233,234). These lysosomal hydrolases degrade a broad spectrum of substrates in the acidic environment of the wound. Up to 80% of the specific lysosomal proteinases cathepsins B, L, and D are actively secreted by inflammatory macrophages (181). The lysosomes contain at least 40 acid hydrolases with a broad range of substrate specificities (205,229,233,234).

Inflammatory macrophages are probably the major source of IL-1α and IL-1β. These molecules are pleiotropic in their actions: their endocrine bacteriostatic functions include induction of fever by resetting the hypothalamic set point (59,65,151,170) and by induction of hepatocyte synthesis of acute-phase reactants such as anti-bacterial serum amyloid A and haptoglobin (50,170,214). IL-1 also induces secretion of another bacteriostatic effector, lactoferrin, which is a chemoattractant for T cells, B cells, neutrophils, and macrophages as well (38,59,170). Many of these effects of IL-1α and IL-1β are duplicated by TNF-α (51,77) Both IL-1 and TNF-α are induced by lipopolysaccharides and by macrophage phagocytosis.

Recent work indicates that there is a dense inter-regulation of growth factor production in macrophages and the other cells in wound foci. The growth factors produced in wound attract more leukocytes to the wound, stimulate antibacterial activity, activate leukocyte production in the bone marrow and trigger a number of positive loops in cells of the wound (153,155,245). In the wound-derived macrophage the macrophage chemoattractants C5a and TGF-β induce macrophage IL-1 production (166,244). These stimulated macrophages produce more TGF-β (7). IL-1 induces further IL-1 polypeptide and mRNA synthesis in

macrophages (59). Macrophage TNF-α induces endothelial cells and fibroblast to produce IL-1 (122,123), and TNF-α and IL-1 induce G-CSF, GM-CSF, and M-CSF in endothelial cells (209). Macrophages themselves synthesize G-CSF, GM-CSF, and M-CSF after stimulation by various wound molecules (187,189,230,245). These colony stimulating factors have paracrine activating effects on inflammatory leukocytes as well as stimulating endocrine hemopoietic effects in bone marrow (153,155,245).

In summary, macrophages play a central role in acute and chronic inflammation. Major secreted effectors are lipid derivatives (PGE series and LTC_4), neutral proteinases and lysozyme, lysosomal acid hydrolases, and regulatory molecules such as IL-1 and TNF, GM-CSF, M-CSF, and G-CSF.

Tumoricidal and Microbicidal Functions

Cytocidal activity is one of the macrophage's major functions. Macrophages kill bacteria, yeast, virus-infected cells, parasites, and tumor cells by several modes as described recently by Lachmann (125). Professional cytotoxic cells such as macrophages secrete three types of killing molecules: "plugs, burning molecules, and poisons." Plugs are pore-forming molecules that intercalate into target organism plasmalemma and kill via osmotic equilibration as well as by loss of small molecules. Macrophages synthesize components of the recognition and amplification phase of the classic and alternate complement pathways. There is a debate as to whether macrophages produce the actual pore-forming protein C9 (176). Although complement components, especially C3, exist in appreciable concentrations in plasma, the local secretion of the early components by macrophages may shield the complement proteinases from high concentrations of plasma inhibitors, as well as protecting adjacent tissue cells (263). The classic pathway is initiated by antibody-antigen complexes, whereas alternate pathway can be directly activated by polyanionic bacterial and yeast cell walls. Since the alternate pathway can be activated independently of antibodies, the

complement components C3-C9 secreted by macrophages may mediate the entire pathway. Synthesis and secretion of complement components by macrophages are stimulated by the targets themselves, by IL-1, and by γ-IFN during specific immune activation (120,222,226). Prostaglandins and histamine decrease complement synthesis (23,69,127,201). Macrophages also secrete lysozyme, lysosomal enzymes, and other secreted proteins in close proximity to the target directed at the sites of ligand-receptor interaction (Werb et al. submitted for publication).

Some microorganisms resist macrophage dissolution and digestion (e.g., tubercle bacilli). Only activated macrophages can kill these microbes. The microbicidial mechanism is by production of 'burning' molecules: reactive oxygen intermediates which oxidize target cell walls. Reactive oxygen intermediates include hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical. These molecules are the hallmark of the activated macrophage (46,137a,165). Activated T-cell-produced IFN-γ has been identified as one 'macrophage activating factor' that stimulates the oxidative burst that produces these intermediates (159,160,161). T cell production of IL-2 augments monocyte-mediated cytotoxicity by reactive oxygen intermediates (142). GM-CSF and M-CSF, macrophage products, autoactivate the macrophages for tumoricidal activity by stimulating the production of reactive oxygen intermediate (93,104,126). Microbicidial and tumoricidal activities of macrophages are induced by factors from activated T cells and enhanced by autocrine factors.

Macrophages produce molecules that poison tumor cells. TNF-α and, with a less broad spectrum, IL-1 kill some tumor cells with receptors for them. The final step in the cytotoxicity induced by TNF-α is the fragmentation of nuclear DNA (27,177). It has been demonstrated by antibody blocking experiments and development of resistant target variants (236) that macrophage tumor cell killing can be mediated entirely by TNF-α. However, activated macrophages produce the cytolytic neutral proteinase that kills some tumor cells (3).

Interferon-β (IFN-β) secreted by macrophages mediates anti-viral effects in uninfected cells. IFN-β_2 production by macrophages is also induced by TNF (117).

It is not clear how, or if, macrophages control the cytotoxic molecules they secrete. Clearly, the macrophage is rather resistant to oxygen radicals. As indicated earlier, macrophages secrete α_2-M, α_1-PI, and C3-inactivator inhibitor, which may attenuate complement proteinases as well as restricting complement action to the locus of its activation. Prostaglandins and enzyme-inhibitor complexes have a negative effect on local complement synthesis by macrophage. Scavenging enzymes such as catalase, and superoxide dismutase limit the range of reactive oxygen intermediates (157). TNF-α and IL-1 kill by receptor-mediated mechanisms affecting only those cells with an unknown derangement of metabolism downstream from those receptors.

In summary, the macrophage secretes digestive enzymes, molecules that form pores or that burn or poison other cells, in order to kill a variety of targets. They control the extent of killing by secreting a variety of inhibitors that stop reactions extracellularly as well as feeding back on the synthesis of killing molecules.

Immune Presentation

Macrophages are important in the afferent or generative arm of the immune response. Macrophages endocytize and digest antigens and then present them to T helper cells in a complex with Ia antigens on the macrophage surface (234). This presentation is required for the generation of immune responses to many T-dependent antigens. This presentation makes the T cells 'competent' by inducing IL-2 receptor expression. IL-1 can act as a cofactor to antigen and Ia antigen stimulate T cells to produce interleukin-2, which stimulates progression into S phase by binding its receptor (150,151). IL-1 may also directly enhance the B cell response in two ways. First, it stimulates B cell differentiation by inducing a pre-B cell line with only cytoplasmic μ chains to express kappa light chains and subsequent surface

immunoglobulin (179). IL-1 also enhances proliferation and immunoglobulin secretion in mature B cells (11,150,155). Stimulated macrophages also produce immunoglobulin production inducing B cell stimulatory factor-2 (BSF-2), which was recently shown to be synonomous with IL-6, plasmacytoma growth factor, IFN-β2, and hepatocyte stimulating factor (79,231,237). Another reported effect of IL-1 on immune cells is the induction of natural killer activity. There is more than one IL-1 gene, and many IL-1 proteins, in all species surveyed (143). The current hypothesis is that all IL-1 species induce similar immune effects. The development of the humoral response is thought to require the secretion of IL-6 and IL-1 by macrophages, as well as expression of class II MHC molecules and processed antigen on the macrophage surface. The development of the humoral immune response requires the secretion of IL-1 and IL-6 by macrophages, as well as expression of class II MHC molecules and processed antigen on the macrophage surface.

The development of a cellular immune response requires macrophage-T cell interaction and is represented by delayed-type hypersensitivity (DTH). This reaction occurs in previously sensitized individuals and requires 48 hours to develop, whereas the immediate hypersensitivity produced by the humoral immune response has subsided by 48 hours. DTH is characterized by MHC class II restricted interaction between the macrophages and T cells which have migrated into the interstitial site of bacterial infection. The macrophages outnumber T cells in these lesions by more than 10:1, but activated T cells are required to trigger macrophage microbicidal activity. A complex of soluble factors may mediate macrophage-T cell communication during DTH. Activated T cells secrete factors which function to attract macrophages, to prevent their departure and to activate their microbicidal activity (22a). Activated T cells secrete γ-interferon which activates macrophages (4,32,167), GM-CSF which activates macrophages (93) and IL-2 which activates macrophages (142). In response, activated macrophages produce IL-1, TNF-α, and M-CSF (104) which further

activate T cells (38,59,65,150) and the macrophages themselves (4,60,65,177,245). Macrophages activated by T cells eliminate interstitial bacteria by several mechanisms (see previous section on microbicidial functions).

The attenuation of immune responses is complex. Loss of stimulus is preeminent, but prostaglandin E may also down-regulate T cell and macrophage interactions (120,220). Other macrophage secreted products implicated in immune attenuation are TGF-β, arginase and thymidine (39,103, 149, 217,219,223) as well as degradation products from macrophage complement and fibrinogen (158). Apolipoprotein E, a major secreted product of inflammatory, but not activated, macrophages may also be immunosuppressive (20,34,49).

In summary, macrophages are essential in the generation of both humoral and cell mediated immune responses. Presentation of antigen on the cell surface and secretion of IL-1 are required for T and B cell activation. Secreted prostaglandins are important in the down regulation of the response.

Debridement and Fibrinolysis

Acute inflammation resolves between three and seven days after wounding. The predominant wound cell in this period is the macrophage. The wound is acidic, filled with dying PMN, fibrin clot fragments, degraded extracellular matrix molecules, and dead and dying bacteria, yeast, and various residues of inflammation. The macrophage is stimulated by these materials (lipopolysaccharides, zymosan, complement C3b-opsonized particles) to secrete uPA (31). Transcription of uPA is induced by IFN-γ through suppression of short lived repressors (47,99). Macrophage uPA is also controlled by M-CSF (134). As stated previously, this serine proteinase has numerous effects. It mediates fibrinolysis by clipping a peptide from plasminogen, which, after a subsequent autoproteolytic cleavage event, cleaves fibrin (31). Plasmin also cleaves and activates other extracellular matrix-degrading proenzymes, such as procollagenase and prostromelysin (180,189), which are secreted by

macrophages. When monocytes first enter an inflammatory site they contain the serine proteinases PMN-type elastase and cathepsin G in their azurophil granules (246) and on their surface (76). As they become macrophages, they gain the capacity to express these enzymes (246,247). In addition, oxygen radicals produced by activated macrophages and PMN have direct effects on connective tissue by direct degradative effects on matrix components, and indirect action through activation of procollagenase and inactivation of α_1PI (35,111).

Metalloproteinases, such as collagenases, stromelysin, gelatinases, and elastase, are secreted by inflammatory, but not resident, macrophages (16,73,102,250,255,256). These macrophage neutral proteinases have a small intracellular compartment and are secreted continuously. Phagocytosis of particles of collagen and other debris by macrophages is a powerful inducer of collagenase and elastase production (29,227,249,255,256). IFN-γ also induces collagenase production by macrophages (243).

Macrophages secrete several proteinases that have separate specificities for collagen. Collagenase cleaves interstitial collagens (types I, II, III, VIII, and IX) into one-quarter and three-quarter fragments similar to other mammalian collagenases (249). This collagenase is a metalloproteinase that is activated by plasmin. A second proenzyme, 95 kDa gelatinase, which cleaves pericellular type V collagen and denatured collagen, is also a metalloproteinase (140,141,239). A third enzyme of 68 kDa cleaves basement membrane type IV collagen (48). In addition, stromelysin degrades many matrix proteins, including type IV collagen and proteoglycans (76,146).

Macrophages also secrete molecules that induce fibroblasts, endothelial cells, synovial cells, and chondrocytes to secrete collagenase (51,52,53,99,156,182,183,206). Although fibroblasts are not a prominent cell in the resolving inflammatory focus, they are an important component of granulation tissue (129,191).

Uptake of fragments of matrix debris such as collagen induces macrophages to secrete IL-1 and PGE_2 (29,53). PGE_2, in turn, stimulates production of collagenase by macrophages (242). IL-1 also induces the metalloproteinase collagenase in synovial cells (50), and fibroblasts (182,183) and IL-1 induces collagenase and stromelysin synthesis in rabbit synovial cells (74). Expression of collagenase is also induced by TNF-α in human synovial and dermal fibroblasts (51) and is repressed by anti-inflammatory glucocorticoids (74,248). Similarly, uPA production by fibroblasts is induced by mitogens and IL-1 (66,128). Collagenase can be expressed by stimulation of fibroblasts with PDGF (21,48) fibroblast growth factor (FGF) (67,98), or (EGF) (43,67,74,146), and by stimulation of endothelial cells with FGF (98). In addition, TGF-β decreases metalloproteinase expression (98). Because PDGF, FGF, TGF-α, and TGF-β are products of macrophages induced by inflammatory stimuli such as lipopolysaccharide (LPS) and lipid loading (189) this provides another pathway for regulation of extracellular matrix turnover during debridement. It seems likely that TGF-β, which reduces metalloproteinase expression and induces the expression of metalloproteinase inhibitor (see below), reverses the earlier phase of cell growth and extracellular matrix degradation in the debriding wound.

The turnover of extracellular matrix by the proteinases is regulated by inhibitors such as the tissue inhibitor of metalloproteinases (TIMP) (61) and by PAI-2 (31,240). Stimulation of macrophages by LPS concomitantly stimulates TIMP and collagenase synthesis (247). This concomitant synthesis may limit the range of the collagenase. Macrophage-generated IL-1 and PDGF also induce fibroblasts to produce more TIMP, and TGF-β can increase TIMP expression while repressing metalloproteinase synthesis (67,156).

Macrophage elastase is a metalloproteinase, whereas neutrophil elastase and the prepackaged elastase in monocyte azurophil granules are serine proteases. Macrophage elastase has limited activity but is able to cleave insoluble matrices of several cell types and is

thought to be pathophysiologically relevant (112,251). Macrophage elastase may act pathologically in generating emphysematous lesions when there is prolonged stimulation or insufficient proteinase inhibitor secretion occurs (15). Elastase is capable of limited proteolysis of IgG, fibrinogen, α_1PI and cartilage proteoglycans (15,16). The cleaved α_1-PI are a potent chemoattractant for PMN (17).

The products of cleavage of extracellular matrix macromolecules generated by macrophage proteinases are phagocytized and further degraded within intracellular lysosomes. Macrophages also actively secrete lysosomal proteinases such as cathepsin L (181). Wound debridement includes killing of viable microbes and dissolution of clot and extracellular matrix molecules followed by phagocytosis and intracellular degradation of remnant molecules.

Macrophages also secrete inhibitors that limit the longevity and scope of proteinase activity, including PAI-2, α_1PI (154,238), α_2M (105), complement inhibitors, and TIMP (18,247). α_2M is a 'trap inhibitor' that closes upon a wide range of specific proteinases after these proteinases act upon it. The α_2M-proteinase complex is then phagocytized via a receptor-mediated process that decreases further proteinase synthesis.

In summary, macrophages are the primary cell type involved in debriding acute inflammatory foci. They mediate debridement by secretion of plasminogen activator, collagenases, and elastase. Stimulated macrophages also secrete α_1PI, PAI-2, α_2M, and TIMP, which regulate the scope of the extracellular proteinases.

Neovascularization and Wound Healing

Wound healing begins during the resolution of acute inflammation. The kinetics and properties of wound healing vary according to the extent of the wound. Wound healing processes replace the cells and extracellular matrix lost during wounding and inflammation.

During the resolution of inflammation, endothelial cells sprout from existing periwound vasculature (72), and periwound fibroblasts migrate into the wound and proliferate. After the first week after wounding, fibroblasts and endothelial cells have filled the wound with loose connective tissue and a dense capillary network, respectively. The mass of proliferating capillaries and fibroblasts and residual macrophages is called 'granulation tissue.' By the end of the second week the capillary network has thinned and collagen fibrosis has increased (129,194). Ablation experiments demonstrate that macrophages, but not PMN, are required for angiogenesis and wound healing (129,130). Others have found that activated macrophages and wound fluid induce wound healing (14,97).

Macrophages secrete a number of chemotactic factors for fibroblasts. These include fibronectin (232) and PDGF (144,196,211). The procoagulants and fibronectin produced by macrophages also contribute to a framework on which the cells migrate (5,172).

Macrophages induce vascular proliferation (116,180), and an overlapping set of secreted macrophage factors induce fibroblast proliferation (87). Basic fibroblast growth factor (bFGF) is synthesized by stimulated macrophages and induces fibroplasia and angiogenesis (13); however, it is secreted by P388D1 macrophages but not by stimulated primary macrophages in vitro (189). It may be released by primary macrophages upon their death, however bFGF was recently cloned and sequenced and has no signal sequence (1,2). It may be released by a novel secretory pathway or through cell death. IL-1 also shares this absence of an identifiable signal sequence (136). IL-1 has limited mitogenic activity for fibroblasts and astroglial cells, but none for endothelial cells (85,133,203). Other factors that induce fibroblast proliferation and fibrosis in vivo or in vitro and are produced by macrophages are PDGF, TGF-β, TGF-α and bombesin (94, 138,144,145,207,211,218,259). Mitogenic transforming growth factors have recently been implicated in several phases of wound healing. TGF-β and TGF-α have been implicated in angiogenesis in vivo (194,207), and TGF-α has

been implicated in epidermal regrowth and formation of granulation tissue in vivo (208). A nonmitogenic angiogenic factor has been isolated from wound fluid (14) and from hypoxic macrophages (hypoxia is a hallmark of wounds) in vitro (116).

Two groups have claimed that TNF-α is angiogenic. One group claims that TNF-α is directly angiogenic for endothelial cells, but because it is not mitogenic for these cells the angiogenesis is limited to capillary sprouting (131). The second group concludes that the angiogenic effect is secondary to the inflammatory effect of TNF-α (71) because of its chemoattractant activity for monocytes and PMN (149).

Macrophages are also prominent in nerve regeneration (174). Macrophages enter a crushed nerve within a few days of the trauma and debride it by degrading myelin (36). Macrophages secrete apolipoprotein E (20,252-254), which is the most abundant protein in injured nerves (108) and may promote growth of glial cells. IL-1 also stimulates NGF synthesis in nerves (135). Macrophages also degrade myelin (36).

The initial phase of wound healing consisting of an increase in cellularity, is followed by a second phase of consisting of an increase in extracellular matrix. Collagen deposition by wound fibroblasts is stimulated by macrophages stimulated by indigestible silica or other materials in vitro or in vivo (109). The fibrogenic activity is probably accounted for by IL-1 and TGF-β (145,194,203). Collagen synthesis is also regulated by IFN-β (64).

The termination of angiogenesis and of proliferation of fibroblasts is as important as is the initiation of these processes. First, most growth factor mRNA is short-lived owing to a conserved sequence in the 3′ untranslated end of the transcript (210). After the growth factor-inducing stimulus ends, the transcript is destroyed within the macrophage and the factors (which are not stored) disappear. Second, macrophages may become retractory to stimuli which trigger growth factor production in the wound. Macrophages may become refractory to many inducers in vitro. PDGF and GM-CSF transcript accumulation ends 12

hours after LPS stimulation, and IL-1 transcript accumulation ends 24 hours after LPS stimulation despite the persistence of LPS in the medium. Third, macrophage products may oppose the actions of growth factors. In certain circumstances PGE and IFN-β can counteract the effects of growth factors secreted by macrophages (81,82,110). Fourth, macrophage products may directly interact with and disable growth factors. Macrophages produce IL-1 inhibitors (132), and both IL-1 and FGF are susceptible to proteolysis by proteinases produced by macrophages (M.J. Banda, unpublished results). Macrophages also produce a TGF-β binding protein that may modulate TGF-β activity (7).

In summary, macrophages deliver several factors that regulate migration and proliferation of fibroblasts and endothelial cells, and collagen deposition by fibroblasts. Products such as PGE and IFN-β may regulate responsiveness to these factors, but the short half-lives of growth factor mRNAs ensure a limitation on growth induction.

Conclusion

Macrophages regulate a number of important extracellular pathways by means of a myriad of secretory products. They are a ubiquitous cell type with distinct tissue-dependent differentiation phenotypes. They mediate important pathophysiological processes by sensing and reacting to environmental stimuli.

Macrophages secrete pleiotropic factors that participate in hemostasis, inflammation, killing, immune presentation, and wound debridement and healing. The secretory pattern can be classified into three broad phenotypes: *resident* macrophages are relatively quiescent, *inflammatory* macrophages secrete a variety of neutral proteinases, and *activated* macrophages secrete fewer proteinases and more cytotoxic molecules (such as reactive oxygen intermediates). Macrophages secrete a number of factors that initiate extracellular cascades: procoagulants, early complement components, neutral proteinases and their activators, and growth factors. These extracellular cascades are attenuated in some cases by

their own end products, but macrophages also secrete negative modulators such as PGE, α_2M, and IFN-α, and IFN-β.

Macrophages are a rich source of questions and answers to researchers in pathophysiology and cell biology. A new technique for single-cell mRNA phenotyping holds the promise of rapid progress in studying macrophgaes in vivo (189,190). The purification of secreted proteins and polypeptides and the ensuing production of cDNA and antibody probes will allow finer dissection of macrophage secretory capacity/function relationships.

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Chapter 2

A Novel Method for Studying mRNA Phenotypes in Single or Small Numbers of Cells

Abbreviations used: GuSCN, guanidine thiocyanate; IL-1α, interleukin-1α; MMLV-RT, Moloney murine leukemia virus reverse transcriptase; NGF, nerve growth factor; PCR, polymerase chain reaction; RT, reverse transcription; Taq, *Thermus aquaticus*.

Biological processes, such as growth control, are often governed by biochemical steps involving mRNA transcripts that are short-lived and have a low copy number. Furthermore, the cells involved in these processes are often available in low numbers from *in vivo* sources. We now report a method that is superior to *in situ* hybridization, RNA blot analysis, and the nuclease protection assay for the study of short-lived, low-copy-number mRNA transcripts. The method consists of a micro-procedure for isolating RNA from one to a few thousand cells and two coupled enzymatic steps: reverse transcription of whole cellular RNA, followed by amplification of the cDNA by a specifically primed polymerase chain reaction to give specific cDNA fragments that can be visualized on agarose gels by ethidium bromide staining. With this method we have detected actin mRNA from a single cell, or <100 cRNA molecules, and have quantified differences in RNA concentrations of $<$ 3-fold. The reverse transcription reaction products can be divided for the polymerase chain reaction, and several mRNA species can be assayed simultaneously. Therefore, we call the method single-cell mRNA phenotyping. This technique is applicable to the analysis of low-copy-number growth factor transcripts in cells in culture and *in vivo*.

The study of growth factor transcription has been slowed by a number of obstacles. Growth factors are generally transcribed in low copy numbers and have a short half-life (1). In addition, often low numbers of primary cells can be obtained from *in vivo* sources. *In situ* hybridization analysis for mRNA in cells in tissue is difficult and insensitive (2). One approach has been to use continuous cell lines to obtain sufficient material for growth factor studies, but immortalization of cell lines is often produced by ectopic expression of the growth factors in question (3).

Two developments have now permitted development of a novel method for RNA analysis. First, a method for cell-free DNA replication was reported (4). This method, the polymerase chain reaction (PCR),[1] involves two oligonucleotide primers that flank the DNA sequence to be amplified, repeated cycles of thermal denaturation of the DNA, annealing of primers to their complementary sequences, and primer extension to give an exponential accumulation of the target fragments. PCR has been used for selective enrichment of DNA sequences and sequence variations and for high efficiency cloning of genomic sequences and has been improved by the use of a newly isolated thermostable DNA polymerase from *Thermus aquaticus* (Taq) (5). In addition, the reverse transcriptase from Moloney murine leukemia virus (MMLV-RT), which produces cDNA from whole cellular RNA mixtures, was recently isolated (6).

We now report that a microadaptation of the guanidine thiocyanate/cesium chloride (GuSCN/CsCl) gradient ultracentrifugation technique (7), followed by MMLV-RT production of cDNA and PCR amplification of specific cDNA subfragments, allows the quantitative assay of multiple transcript types from a low number of total transcripts. We have found that (a) a single cell yields a signal for β-actin mRNA; (b) as few as 100 cRNA transcripts can be detected; (c) each cDNA molecule can generate 10^9 to 10^{10} PCR fragments; and (d) < 3-fold differences in mRNA can be resolved after PCR amplification of

cDNA.

MATERIALS AND METHODS

Materials

Cells were thioglycollate-elicited peritoneal macrophages (8) harvested on day 4 after stimulation from CF1 mice (Charles River Laboratories). Hep-SK cells were obtained from the American Type Culture Collection. Restriction endonucleases were obtained from BRL and Promega Biotech and used according to the manufacturer's instructions. Taq DNA polymerase and DNA Thermocycler programmable heating block were obtained from Perkin Elmer-Cetus Corporation. MMLV-RT was purchased from BRL. cDNA probe for chick actin was a gift of M. Kirschner (9). Primers were purchased from UCSF Biomolecular Resource Center or were provided by Cetus Corporation. [^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham. SeaKem ME and NuSieve GTG agaroses were purchased from FMC Corporation. DNA 1-kb molecular weight standards were purchased from BRL, and Zeta-bind nylon membranes were purchased from BioRad. Apparatus for agarose gel electrophoresis was purchased from American Bionetics. CsCl was purchased from Pharmacia, and GuSCN was purchased from Fluka. Oligo-dT primers and dNTPs (dATP, dCTP, TTP, dGTP) were purchased from Pharmacia. RNAsin and T7 polymerase were purchased from Promega Biotech. Acetylated bovine serum albumin was purchased from Sigma. Nerve growth factor (NGF) cRNA, produced from an SP6 clone, was a gift of Dr. Dennis Clegg. The poly A$^+$-pBR322-IL-1α cRNA was synthesized from a plasmid template (D. Mark, unpublished results) by T7 polymerase, purified by oligo-dT chromatography, and quantified by absorbance at 260 nm.

The mouse β-actin (10) primers were (5′-primer) 5′-GTGGGCCGCTCTAGGCACCA-3′, 5′ = residue 125, and (3′-primer) 5′-

TGGCCTTAGGGTGCAGGGGG-3′, 3′ = residue 264. The mouse NGF (11) primers were (5′ primer) 5′-CCAAGGACGCAGCTTTCTAT-3′, 5′ = residue 246, and (3′ primer) 5′-CTCCGGTGAGTCCTGTTGAA-3′, 3′ = residue 649.

RNA Preparation

RNA was prepared by a microadaptation of the GuSCN/CsCl gradient ultracentrifugation method (7). Briefly, macrophages (as few as one cell per microtiter well, as determined by microscopic examination) were cultured, washed with 0.9% NaCl, lysed in 100 μl of 4 M GuSCN containing 10-20 μg *Escherichia coli* ribosomal RNA as carrier, layered over 100 μl 5.7 M CsCl, and centrifuged for approximately 20 x 10^6 *g*-min/cm of gradient length, in a Beckman TL-100 tabletop ultracentrifuge or in a Beckman Airfuge. RNA was ethanol precipitated, centrifuged, and then washed in 80% ethanol. The dried RNA pellet was redissolved directly in reverse transcription (RT) buffer (described below) or in diethyl pyrocarbonate-treated H_2O containing RNAsin.

Reverse Transcription

RNA was reverse transcribed into cDNA essentially as previously described (6). RNA (from 1 cell equivalent to 1 μg) was incubated at 37°C for 60 min with a mixture of 100 U of MMLV-RT and the following reagents: 0.2 μg oligo-dT primer, 3 mM $MgCl_2$, 10 mM Tris-HCl buffer, pH 8.3, 75 mM KCl, 1 μg acetylated bovine serum albumin, 0.5 mM dNTP, and 5 U of RNAsin in 10 μl volume. The RT can be repeated by addition of 50 U of fresh MMLV-RT after a 93°C, 5-min denaturing step, followed by flash cooling to 4°C.

Polymerase Chain Reaction

PCR was performed essentially as previously described (5). A small portion of RT products (1 μl) was mixed with 1 U of Taq DNA polymerase, 50 pmol of 20-25 base-long oligonucleotide 5′ and 3′ sequence-specific primers, in a buffer containing 10 mM Tris-HCl,

2.5 mM $MgCl_2$, 50 mM KCl, 5 μg acetylated bovine serum albumin, pH 8.3, in 50 μl volume. The mixture was overlaid with mineral oil to prevent evaporation and then amplified by PCR in a repeated 3-temperature cycle on the Thermocycler programmable heating block. The temperature used in the annealing cycle was varied in proportion to the melting temperature of the primers (given GC pairs contribute 4° and AT pairs contribute 2° to the melting temperature); temperatures were also lowered when using primers in other species for which the DNA sequence was not known. When primers were designed from sequences across species, annealing temperatures from 25°-55°C were tried. For samples to be amplified for >60 cycles of PCR, 1 U of Taq polymerase was added at 60 cycles.

Agarose Gel Electrophoresis

A portion of the PCR mixture (5 μl) was added to 4 μl of loading dye mix and electrophoresed in an 80 V constant-voltage field in 3% NuSieve GTG/1% SeaKem ME agaroses until the bromphenol blue dye front had migrated 6 cm. Gels were stained for 10 min in ethidium bromide and destained in H_2O for at least 30 min.

DNA Blot Analysis

DNA separated on agarose gels was transferred to Zeta-bind nylon membranes as previously described (12). Gels were incubated for two 10-min periods at ambient temperature with 0.25 M HCl, incubated overnight in 0.4 M NaOH, and rinsed in 2x SSC (1x SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). cDNA probes were prepared by random priming (13). Zeta-bind transfers were hybridized by standard techniques (13) and washed at 60°C for 60 min in 0.2x SSC containing 0.1% SDS before autoradiography.

Restriction Enzyme Analysis

PCR products were ethanol precipitated and resuspended in the appropriate restriction enzyme buffer. Three-quarters of the resuspended products were digested, and 5 μl of this

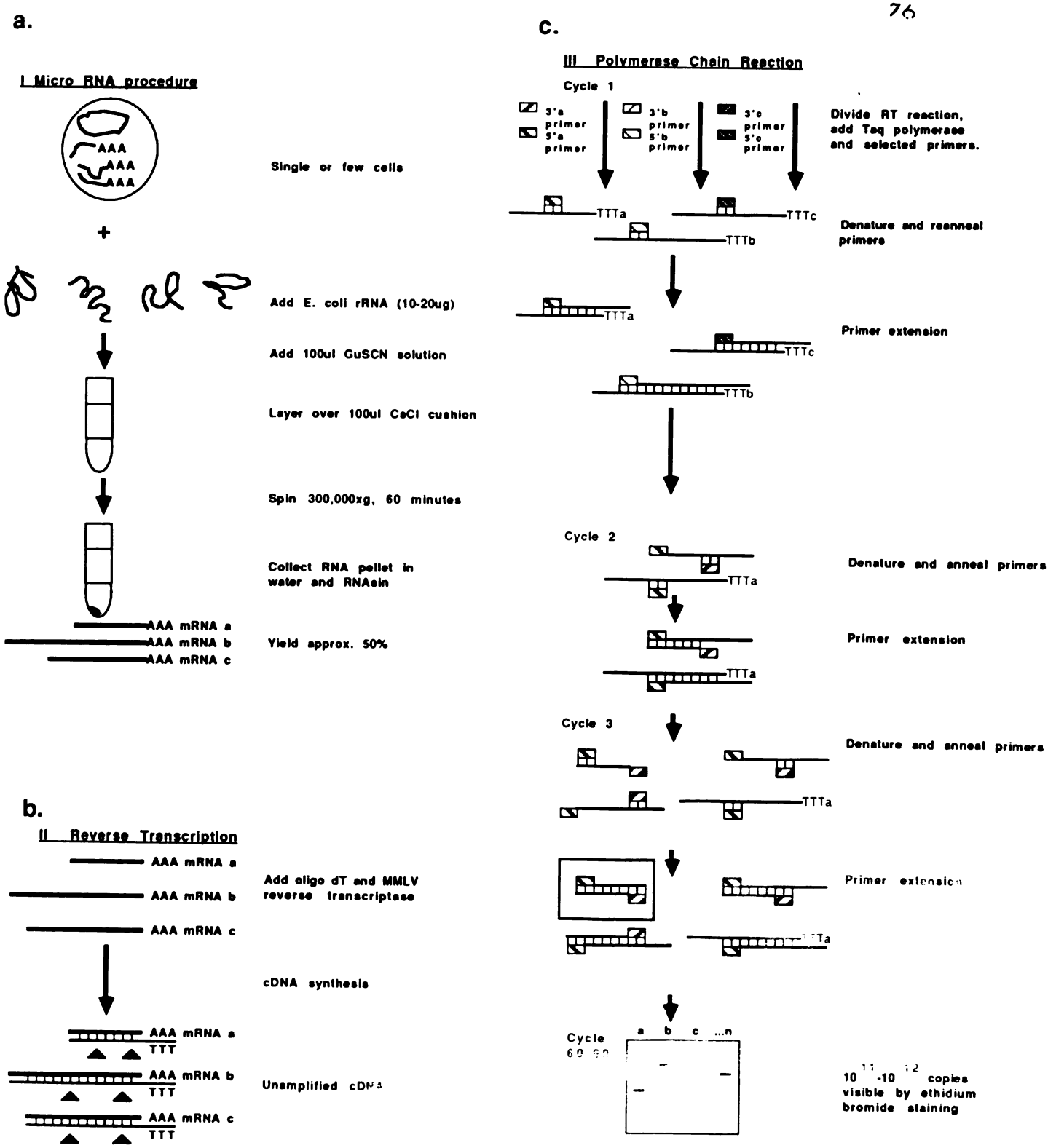
Quantitative Analysis

Two procedures were used to obtain quantitative data: (a) An RT mixture from thioglycollate-elicited macrophages was mixed with PCR reagents (those previously described plus 100 μCi of [^{32}P]dCTP, spec. act. 3000 Ci/mmol) in 2-μl aliquots after 1/100, 1/10, or no dilution. Samples of this mixture (5 μl) were removed after 35, 40, 45, 50, and 55 cycles of the PCR, mixed with loading dye, and then separated on a 1.5-mm-thick 5% polyacrylamide/7 M urea gel (13) and run at 20 mA constant current until the bromphenol blue front had migrated 9 cm into the separating gel. The gel was autoradiographed, appropriate bands were cut from the gel, and radioactivity was determined by scintillation fluorography. Data were plotted by exponential curve fitting using an Apple Cricket Graph® program. (b) Samples of pBR322-IL-1α cRNA were serially diluted by 3-fold and mixed with 1 μg of Hep-SK RNA so that the RT mixture contained 3 x 10^2 to 1.3 x 10^7 copies of cRNA. After RT, the cDNA was amplified by PCR for 40 cycles in the presence of [^{32}P]dCTP to follow incorporation into the specific cDNA fragment band.

RESULTS AND DISCUSSION

A diagram outlining the steps in the RNA phenotyping analysis is shown in Fig. 1. The first step was to obtain the mRNA, which can be done by any conventional procedure for obtaining total or cytoplasmic RNA from tissue or large numbers of cells (13) or by a micro-method for obtaining total RNA from 1 to 10^5 cells (Fig. 1a). The RNA was then reverse transcribed to prepare cDNA (Fig. 1b). This cDNA preparation was divided as desired for analyzing specific mRNA transcripts. For each mRNA species to be analyzed, a designated sequence (Fig. 1b, arrowheads) was selected, and 5′ and 3′ sequence oligonucleotides were prepared according to the criteria outlined in Table I. The primers were added to the cDNA preparation along with Taq polymerase and dNTPs. The cDNA was then thermally denatured, followed by cooling to allow annealing of primers, and the temperature was then

Figure 1. Steps involved in mRNA phenotyping in low numbers of cells. (a) Purification of whole cellular RNA from small numbers of cells. (b) Reverse transcription using MMLV-RT. The arrowheads indicate the designated sequences for amplification by PCR. (c) Early PCR cycles and the visualization of PCR products in an agarose gel. Note that cycles 2 and 3 focus on amplification of cDNA "a" only, but the schematic of the agarose gel at bottom represents cDNA "a", "b", and "c".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Add 100ul GuSCN solution
Layer over 100ul CsCl cushion
Spin 300,000xg, 60 minutes
Collect RNA pellet in water and RNAsin
AAA mRNA a
AAA mRNA b
AAA mRNA c
Yield approx. 50%
b.
II Reverse Transcription
AAA mRNA a
AAA mRNA b
AAA mRNA c
Add oligo dT and MMLV reverse transcriptase
cDNA synthesis
AAA mRNA a
TTT
AAA mRNA b
TTT
AAA mRNA c
TTT
Unamplified cDNA
c.
III Polymerase Chain Reaction
Cycle 1
3'a primer
5'a primer
3'b primer
5'b primer
3'c primer
5'c primer
Divide RT reaction, add Taq polymerase and selected primers.
TTTa
TTTc
TTTb
Denature and reanneal primers
TTTa
Primer extension
TTTc
TTTb
Cycle 2
TTTa
Denature and anneal primers
Primer extension
TTTa
Cycle 3
Denature and anneal primers
TTTa
Primer extension
TTTa
Cycle
a b c ...n
60 90
10 -10 copies
visible by ethidium bromide staining

Figure 1

increased to the optimum for primer extension by Taq polymerase (Fig. 2). In the first PCR cycle only the 5′ primers annealed to the cDNA from the RT (Fig. 1c); after primer extension a cDNA sequence extending from the 5′ primer for various lengths, up to and including the 3′ end of the cDNA, was obtained. In the second cycle, the 3′ primers annealed to the DNA synthesized in the first cycle, and the 5′ primers annealed to the original cDNA; after primer extension the first copies of DNA of the exact size of the designated sequence flanked by the primers were produced. In subsequent cycles, this exact-size DNA became dominant, so that by 30 to 90 cycles the 10^{11} to 10^{12} copies of cDNA, visible by ethidium bromide staining, contained only this specific sequence (Fig. 1c). The intensity of the ethidium bromide signal was related to the number of fragments in each well over a ~ 20-fold dilution range (data not shown). The ideal equation describing the generation of fragments in the PCR is exponential, similar to those for describing bacterial growth and compound interest:

$$N = N_0(\text{eff.})^n,$$

where N is number of amplified fragments, N_0 is number of input cDNA molecules, eff. is efficiency (1-2 is equivalent to 0-100%), and n is cycle number. However, empirically we have found that cDNA generates fragments efficiently and exponentially only during the first 10 to 20 cycles of the PCR. PCR cycles 30-60 are often only 10% efficient (Fig. 3). These data also show that the theoretical equation does not describe the generation of the fragments. Moreover, extrapolation of the data by curve fitting does not work and shows that small changes in the efficiency of reaction make it impossible to predict N_0 from late cycles, except with the use of a standard as shown below.

A single cell with approximately 1,000 β-actin transcripts (14) generated a signal after RT-PCR that was detectable by ethidium bromide staining after 65 PCR cycles (Fig. 4). With increasing actin mRNA input from 10^2 and 10^4 macrophages, the signal appeared at earlier

TABLE I. Criteria for Choosing Oligonucleotide Primer Pairs for RNA Phenotyping Analysis

1.	Target mRNA sequences should be ~ 0.2 to 0.5 kb in length
2.	For most efficient reverse transcription, target sequences should be toward the 3′ end of the coding region of the mRNA sequence. In general the 3′ untranslated region is not used because of the lack of conservation of these between species
3.	Target sequences should contain an intron/exon border so that genomic DNA and mRNA can be distinguished
4.	Target sequences should contain a diagnostic restriction endonuclease cleavage site to permit analytical validation of fragments
5.	Melting temperature of the two oligonucleotide sequences should be similar and close to the extension temperature in the assay
6.	Target sequences should be within available cDNA probes for validation by DNA blot analysis

Figure 2. The four stages in the operation of the programmable heating block for PCR. The 3-temperature PCR cycle is repeated 30 to 90 times. The PCR kinetics are: a=dimer denaturing (95°C, 30 sec); b=primer-template reannealing (50-70°C, 30 sec); c=primer extension (72°C, 90 sec).

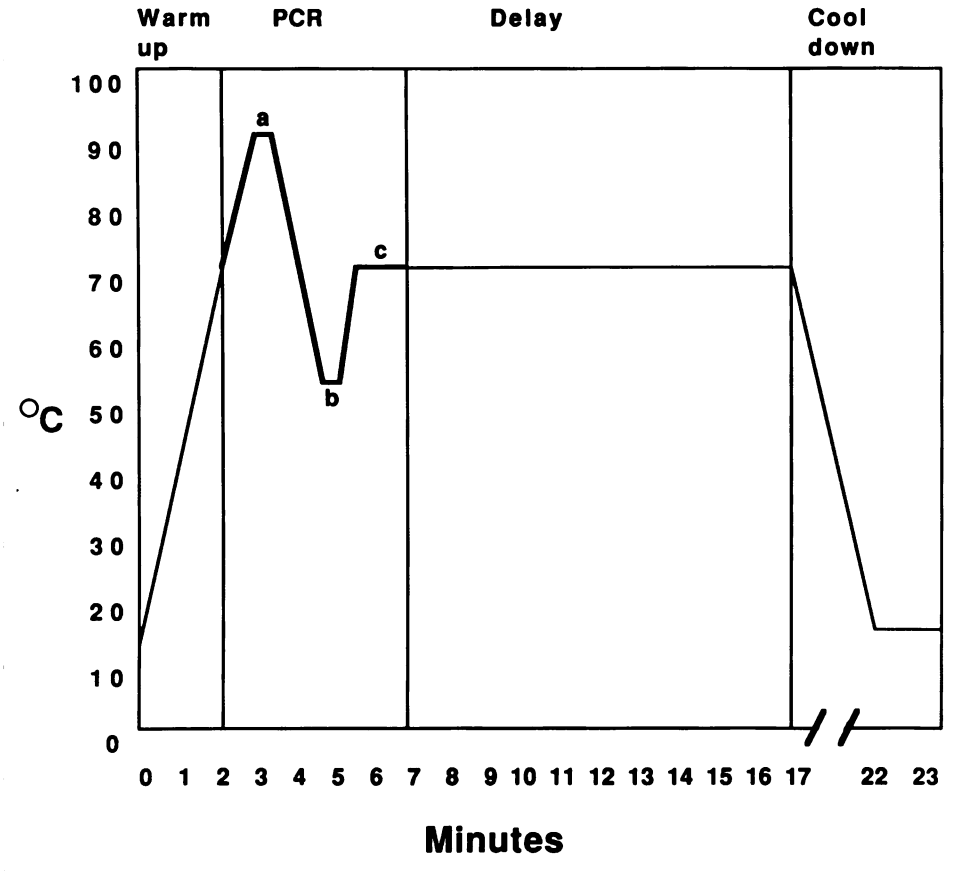
Warm up
PCR
Delay
Cool down
100
90
80
70
60
50
40
30
20
10
0
°C
a
b
c
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 22 23
Minutes

Figure 2

Figure 3. Quantification of differences in input actin mRNA by sampling at various cycles. The efficiency of the generation of PCR products declines after the first 10 to 20 cycles. Note that 10-fold differences in input cDNA result in resolvable differences in output specific signal at all time points measured. These data were obtained by polyacrylamide gel electrophoresis of ^{32}P-labeled cDNA products of PCR as described in Materials and Methods. (a) Autoradiogram of gel. The three sections are 1/100, 1/10, and undiluted mRNA preparations, respectively, sampled at 35, 40, 45, 50, and 55 cycles; (b) cpm of each band of the gel shown in (a).

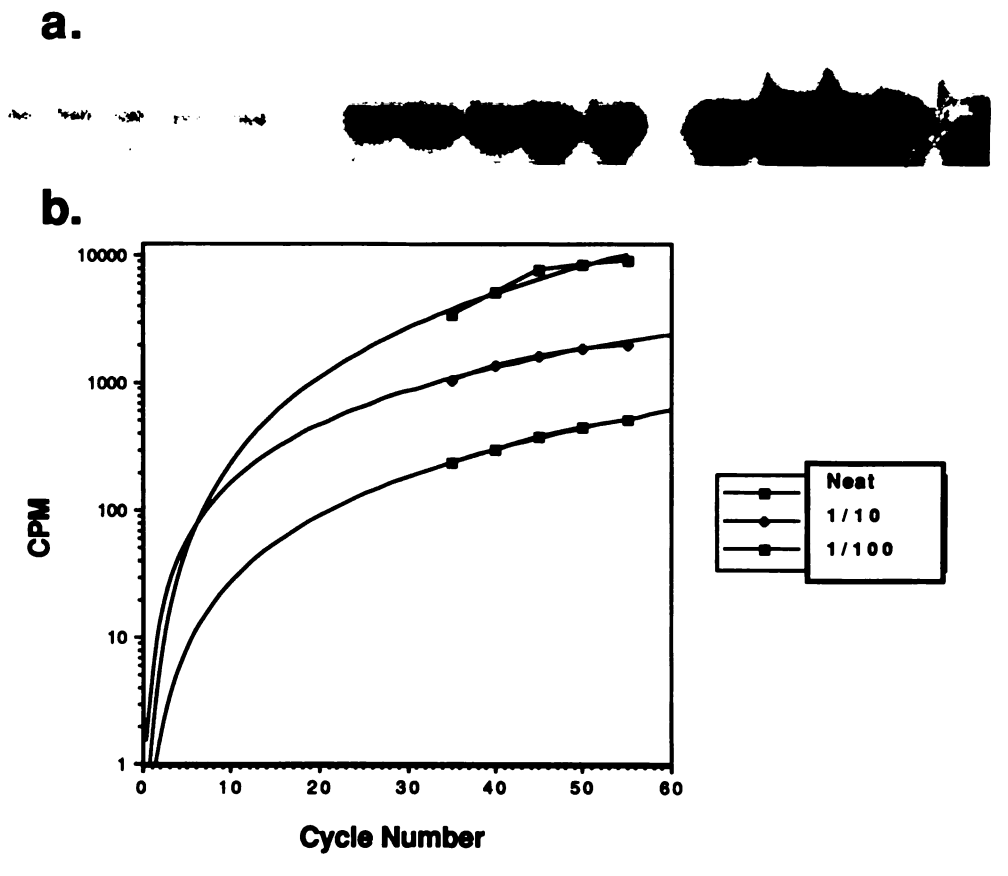
a.
b.
10000
1000
100
10
1
CPM
0
10
20
30
40
50
60
Cycle Number
Neat
1/10
1/100

Figure 3

Figure 4. Generation of a signal detectable by ethidium bromide staining from a single cell. RNA from 1, 10^2, and 10^4 macrophages was reverse transcribed, and the PCR analysis was performed with actin oligonucleotide primers. PCR products were separated by agarose gel electrophoresis, and the gel was stained with ethidium bromide. (a) Diagram of target β-actin sequence between primers (boxes). The position of an intron (triangle) and the position of the *Bgl*II restriction cleavage site are shown. (b) Initial signal validation by size (arrowhead). Numbers at the top are PCR cycles. (c) Further signal validation by restriction enzyme cleavage of β-actin fragment with *Bgl*II (Cut). DNA molecular weight standards (in bp) are shown at left. Arrowheads indicate uncut (top) and cut (bottom) β-actin fragments.

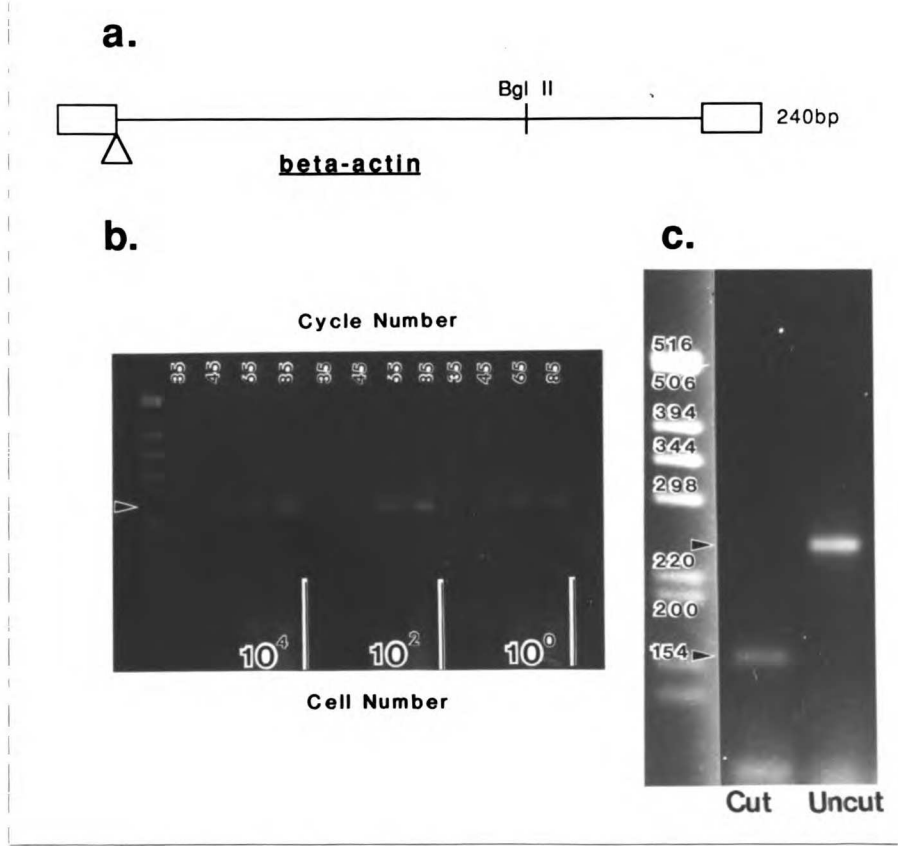
a.
Bgl II
240bp
beta-actin
b.
Cycle Number
Cell Number
10⁴
10²
10⁰
c.
516
506
394
344
298
220
200
154
Cut
Uncut

Figure 4

cycles. At large cycle numbers there was some variability in the strength of the ethidium bromide signal for individual samples, and thus the incorporation of [^{32}P]dCTP into the PCR-generated fragments was the preferred method for quantitative analysis. Signals were validated by four means: (a) the size of the fragment; (b) the presence of diagnostic restriction sites (Fig. 4a); (c) detection by cDNA probes; and (d) direct or indirect sequencing. The results from analysis of a single cell suggested that PCR is less efficient when cDNA mixtures, compared with genomic DNA mixtures, are used. Because the input number of cDNA molecules must be in the range of 1-1,000, we estimated (using the equation given above and a 10^{12}-fragment detection limit) that the PCR is 35-55% efficient when cDNA mixtures are used as templates. The PCR has previously been reported to be 67-90% efficient when genomic mixtures are used (4,5). This discrepancy in efficiency is currently under investigation.

To test the sensitivity of the RT-PCR procedure, we used synthetic NGF cRNA produced from a plasmid containing the SP6 polymerase promoter. We have found that 100 copies of input cRNA can be detected by this method (Fig. 5). This agrees with the result from the single-cell experiment shown in Fig. 4. In fact, because the input cRNA was not polyadenylated, the 3′ primer was used for priming the RT. Because the latter reaction is known to have a lower efficiency than oligo-dT priming, the sensitivity of the procedure may approach 1 copy of input mRNA with oligo-dT primer. Under ideal conditions the MMTV-RT is 10-50% efficient (13).

A difficulty in quantification is suggested by the equation we have been using ($N=N_0$ $(eff.)^n$). A small difference in efficiency could lead to large differences in N. Ideally, small input (N_0) mRNA could be quantified most accurately by using an exogenous cRNA with the same sequence (from the 5′ primer to the poly A tail) as the endogenous mRNA in question. The exogenous cRNA would be diluted serially as an internal standard, and then the amount

Figure 5. Detection of a threshold of 100 molecules of NGF cRNA after RT-PCR, followed by agarose gel electrophoresis and ethidium bromide staining after 60 cycles of PCR.

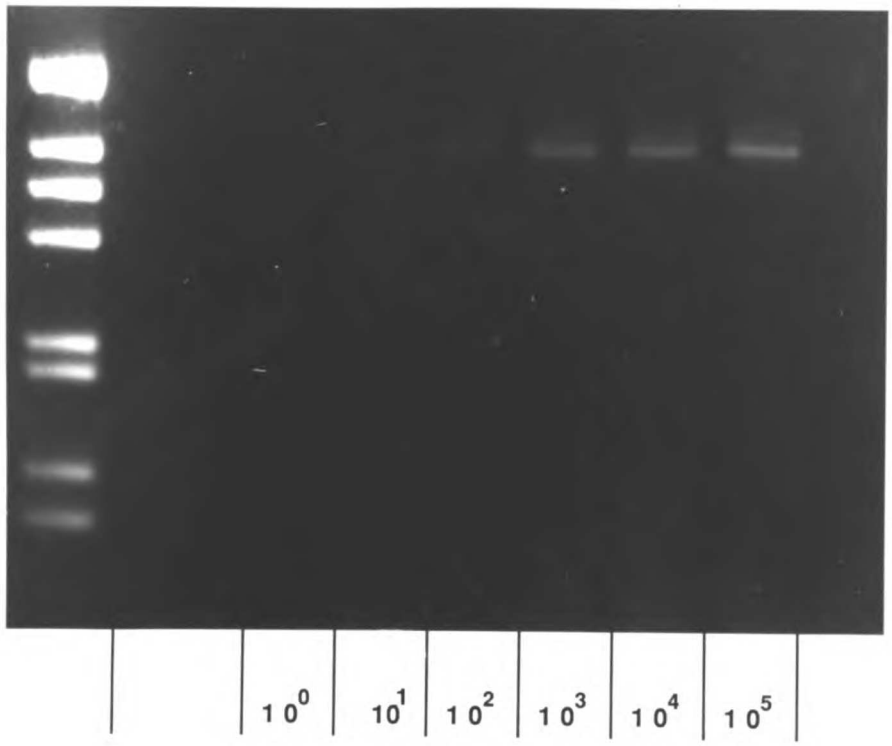

Figure 5

of endogenous mRNA could be extrapolated from the equation of the line. For this endogenous standard to succeed, we first needed to analyze the resolving power of the RT-PCR procedure. In the experiment shown in Fig. 6, 6.5 x 10^3 to 1.3 x 10^7 copies of specific cRNA were used in the RT-PCR in 3-fold dilutions. The results demonstrate that the RT-PCR can resolve < 3-fold differences in N_0 cRNA, with the greatest sensitivity at low N_0. These data will allow us to construct standard curves in an attempt to extrapolate a copy number for the low-copy N_0 endogenous mRNA. The PCR was terminated at 40 cycles in this experiment, but an increase to 60 cycles can result in a minimum signal 100 times lower than the 6,500 copy threshold in Fig. 6. Input RNA present in samples of tissue in which input cell number cannot be determined accurately can be measured by RNA blot analysis of the cellular ribosomal RNA with cDNA probes for vertebrate 28S and/or 18S rRNA. This method can easily detect the rRNA in 2-10 cells (unpublished observation).

The combination of techniques for reverse transcribing mRNA into cDNA by using MMLV-RT, and amplifying specific cDNA segments > 10^9-fold (using Taq DNA polymerase in the PCR), has allowed us to develop a method for phenotyping mRNA in low numbers of cells or with low amounts of mRNA. Some of the advantages and disadvantages of the method are listed in Tables II and III. The major advantages of this novel RNA phenotyping procedure are the ease and rapidity with which mRNA transcripts from limited material can be unambiguously demonstrated. The simultaneous analysis of up to 10 different mRNA species in a single small sample of RNA is possible (15). Specific detection of rare mRNA species such as those for growth factors is possible (15,16). By specific primer design, alternatively spliced mRNAs can be determined. With careful design (e.g., use of conserved amino acids with low degeneracy of codons), primers often work for several species (e.g., human PDGF-A in mouse [15]), even when the species-specific cDNA sequences have not been determined. The PCR fragments so generated can be sequenced and also used as

Figure 6. Resolution of 3-fold differences in input (N_0) pBR322-IL-1α cRNA after PCR amplification of cDNA. Samples were serially diluted, then subjected to RT-PCR. The ^{32}P-labeled cDNA products were separated by gel electrophoresis and the radioactivity was determined.

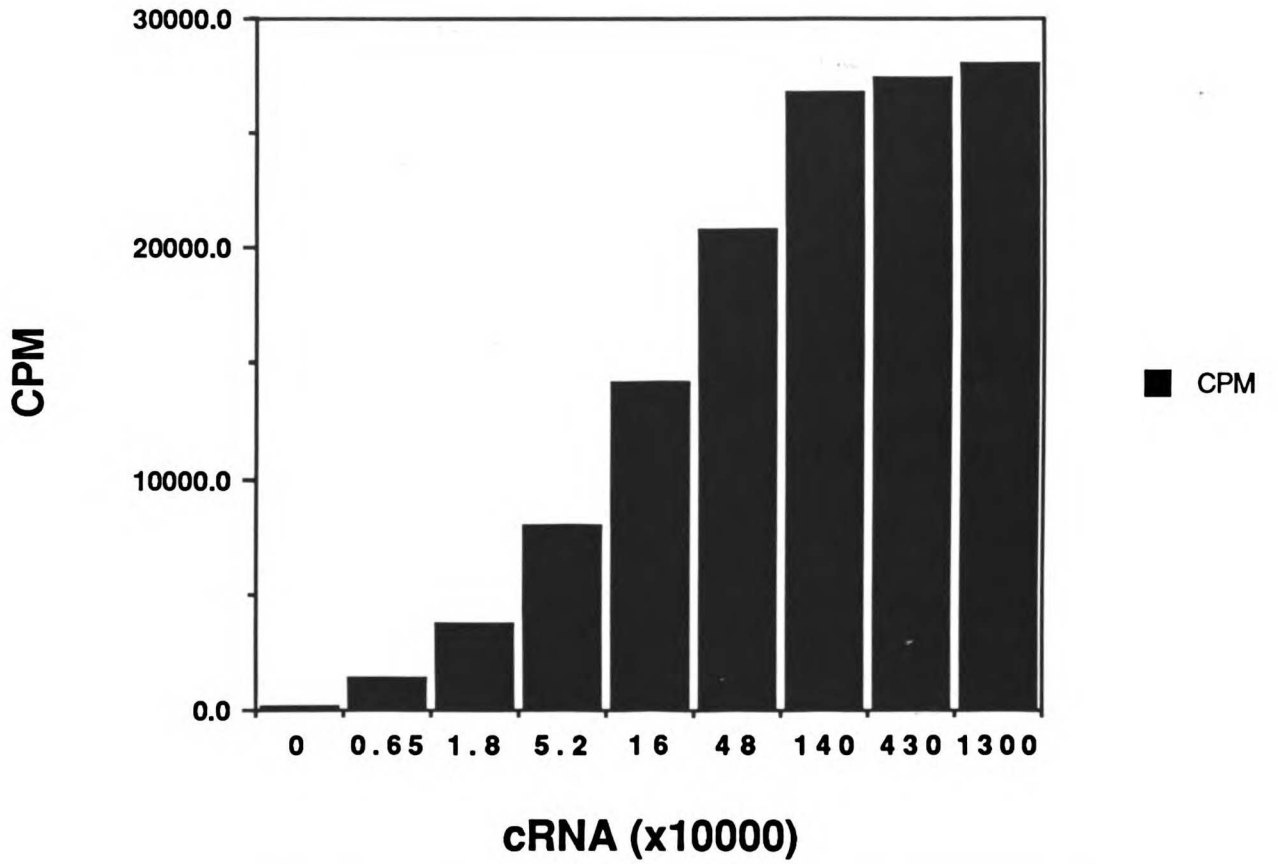
30000.0
20000.0
10000.0
0.0
CPM
0
0.65
1.8
5.2
16
48
140
430
1300
cRNA (x10000)
CPM

Figure 6

TABLE II. Advantages of RNA Phenotyping Method

1. RAPID. Results in < 1 day.
2. SENSITIVE. <100 mRNA molecules detected.
3. LOW NUMBERS OF CELLS. RNA from 1 to a few thousand cells can be analyzed.
4. mRNA PHENOTYPING. >10 different mRNA species within a single sample can be analyzed.
5. RARE mRNA. Specific detection is possible.
6. RESOLUTION. < 3-fold differences can be determined.
7. ALTERNATIVE SPLICING. Easy analysis.
8. MULTIPLE SPECIES. Primers often work across species.
9. RAPID cDNA CLONING. Preparation of species-specific probes is possible.
10. RAPID SEQUENCING. Amplified sequences can be sequenced directly.
11. SAFE. Analysis does not require ^{32}P.
12. PROBES. Rapid preparation of high specific activity probes.
13. EASY. Many samples can be analyzed simultaneously.
14. RELATED MOLECULES. Easy discrimination between molecules.

TABLE III. Disadvantages of RNA Phenotyping Method

1. Does not give mRNA sizes directly.
2. Sensitive to mRNA secondary structure.
3. Does not give three-dimensional information about transcript distribution in cells and tissues.
4. Quantification takes effort.
5. Less resolution (3:1) than other methods.
6. Some sequence information needed.
7. Error rate is high (1/6000) for Taq polymerase.

homologous cDNA probes for other types of RNA analysis (17). Specific primer design or specific restriction endonuclease analysis allows rapid analysis and discrimination of closely related molecules in very small samples (17). Simultaneous analysis of several dozen samples differing in either input RNA and/or primer pairs is possible.

The major disadvantage of this method is that the size of mRNA species is not a direct result of the analysis. Knowledge about alternative splicing patterns is needed to circumvent this problem. As is true for any reaction involving reverse transcription, the technique is sensitive to secondary structure in mRNA. Of > 30 primer pairs designed, a few have been unsuccessful even for abundant mRNA species. Not all primer pairs work with the same efficiency or reliability. Quantitative analysis is cumbersome and difficult, although semiquantitative data can be obtained readily with dilution curves. Although this RT-PCR procedure can be more sensitive than *in situ* hybridization, and has shown the presence of mRNA transcripts not found previously by the latter procedure, it does not give three-dimensional information about transcript distribution in cells and tissues. Good sequence homology is needed to cross species. Although the error rate for Taq polymerase has been estimated at about 1/6000, only one base pair error has been detected in sequencing about 5000 bp of known sequence (17). However, this could present a problem in analysis of point mutations in transcripts.

The potential exists for obtaining these data quantitatively. The RNA phenotyping procedure is superior in ease, speed, sensitivity, and resolution to mRNA analysis by *in situ* hybridization, RNA (Northern) blots, and nuclease protection assays. In other work, we have used this procedure to demonstrate growth factor transcripts in macrophages stimulated by inflammatory agents in culture and in macrophages obtained directly from healing wounds *in vivo* (15). We have also used this method to analyze the regulation of maternal and embryonic transcripts for growth factors (16) and the regulation of proteolytic enzymes (17) in single

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Chapter 3

Wound macrophages express TGF-α and other growth factors in vivo: Analysis by mRNA phenotyping.

The presence of macrophages is required for the regeneration of many cell types during wound healing. Macrophages have been reported to express a wide range of mitogenic factors and cytokines, but none of these factors has been demonstrated to sustain all the wound healing processes in vivo. It has been suggested that transforming growth factor-α (TGF-α) may mediate angiogenesis, epidermal regrowth, and formation of granulation tissue in vivo. We report here that macrophages isolated from a wound site, and not exposed to cell culture conditions, express mRNA transcripts for TGF-α, TGF-β and platelet-derived growth factor A-chain. We determined the expression of these transcripts by using a novel method for RNA analysis in which low numbers of mouse macrophages were isolated from wound cylinders, their RNA was purified and reverse-transcribed, and the cDNA was amplified in a polymerase chain reaction primed with growth factor sequence-specific primers. This single-cell RNA phenotyping procedure is quantitative and rapid, and mRNA transcripts from limited material can be unambiguously demonstrated, with the simultaneous analysis of several mRNA species. Macrophages from wounds expressed TGF-α antigen, and wound fluids contained TGF-α. Elicited macrophages in culture also expressed TGF-α transcripts and polypeptide in a time-dependent manner after stimulation with modified low-density lipoproteins and lipopolysaccharide endotoxin, which are characteristic of the activators found in injured tissues. TGF-α constituted a major mitogenic activity produced by stimulated cultured macrophages. The analysis of transcripts from a single cell or a few cells obtained in vivo has provided the first direct evidence that wound macrophages synthesize multiple growth regulatory factors, and implicates these cells in the necessary repair processes in wound healing through their expression of TGF-α.

When tissues are damaged, a complicated process of healing takes place, the result of which is the replacement of dead tissue and fibrin with a scar. Macrophages are central to the

wound healing response, which requires the proliferation and migration of several regenerating cell types (*1*). In addition to debridement by macrophages, there is an ingrowth of blood vessels from the surrounding connective tissue, the proliferation of fibroblasts, which produce collagen, and the rapid proliferation and migration of epithelial cells over the broken surface. Ablation of macrophages slows the wound healing response (*2*). Previously characterized mitogenic factors derived from macrophages in culture include interleukin 1 (IL-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), colony-stimulating factors (for monocytes, M-CSF, for granulocytes, G-CSF, and for granulocytes and monocytes, GM-CSF), bombesin, and transforming growth factor-β (TGF-β) (*3,4*). Tumor necrosis factor-α, an inflammatory mediator produced by macrophages, was recently reported to be angiogenic (*5*), but others have concluded that this effect is indirect (*6*). Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) bind to the EGF receptor with the same affinity (*7*). It has recently been reported that TGF-α or EGF acts through a direct, noninflammatory mode to direct the wound healing processes in vivo (*8*). Wound fluid, conditioned by macrophages and other cells in vivo, is a rich source of mitogenic and angiogenic activity (*9*). It is not, however, clear which growth factors are actually expressed in vivo, and whether the macrophages recruited to these sites are the source of these polypeptides. We undertook a study to determine whether wound macrophages express growth factors in vivo.

To test our hypothesis, we developed a method for assaying the transcriptional phenotype of small numbers of cells or small amounts of mRNA. This method is superior in ease, speed, sensitivity, and resolution to RNA analysis by *in situ* hybridization, RNA blot analysis, and the nuclease protection assay for the study of short-lived, low-copy-number mRNA transcripts. It can be performed in 1-2 days from cell to analysis. The method consists of a micro-procedure for isolating RNA from one to a few thousand cells, followed by

two coupled enzymatic steps (Fig. 1A). The whole cellular RNA is first reverse transcribed, and then cDNA subfragments are amplified by specifically primed polymerase chain reactions. Each specific cDNA subfragment can be visualized on agarose gels by ethidium bromide staining. Since several mRNA species can be assayed simultaneously, we call the method "single-cell mRNA phenotyping."

Glass-adherent cells (~ 1-2 x 10^2/cylinder, 50-80% macrophages) were isolated from subepidermal wound cylinders (*9*) 6 days after implantation in mice; the RNA was purified and reverse-transcribed, the cDNA was divided, and cDNA subfragments were amplified by sequence-specific primers (Fig. 1A, B; Table 1). Products of the combined reverse transcription-polymerase chain reaction (RT-PCR) were fractionated by electrophoresis in agarose, stained with ethidium bromide, and validated by matching predicted size (Fig. 2A) by means of restriction enzyme analysis (Fig. 2B) or Southern blot analysis (data not shown). The method resolves 3-fold differences in input RNA over a range greater than 2 orders of magnitude (Fig. 2C). Therefore, an exogenous poly A^+ cRNA dilution series can be used as an internal standard to quantify endogenous mRNA transcripts of low copy number (Fig. 2C). With this method we can detect β-actin in a single peritoneal macrophage (Fig. 2D) and in less than 100 copies of cRNA transcripts (*10*).

We found that the adherent cells isolated from wound cylinders contained transcripts for TGF-α, TGF-β, PDGF-A, and EGF, and IGF-I (Fig. 3A). IL-1α was found in 3 out of 6 wound cylinders preparations (Fig. 3A) and was expressed by cultured macrophages stimulated with lipopolysaccharide endotoxin (LPS) (Fig. 2A). We did not, however, find EGF transcripts in macrophage cell lines or in highly purified cultured macrophages (data not shown), suggesting that EGF was the product of a contaminating cell type. Fibroblasts synthesize NGF transcripts in vitro so the absence of NGF transcripts indicates the absence of contaminating fibroblasts. In parallel experiments, we analyzed adherent wound cylinder cells

Figure 1 (**A**) Reverse transcription-polymerase chain reaction (RT-PCR) method for phenotyping macrophage mRNA. RNA was purified by a modification of the guanidine thiocyanate (GuSCN)/CsCl gradient technique (*19*) and reverse-transcribed by using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-dT primers (*20*). Resultant cDNA products were divided, and fragments of cDNA were amplified during 60-90 cycles of polymerase chain reaction (PCR) (*21*) on a Perkin-Elmer Cetus PCR 1000 Thermocycler. (**B**) Structures of PCR-amplified fragments. Rectangles at fragment ends indicate 5 ′ and 3 ′ sequence-specific 20-24 base-long oligonucleotide primers (see Table 1 for sequences), triangles indicate introns, and vertical lines indicate restriction sites. The length of the fragment generated from cDNA is shown at the right. Any genomic DNA contamination of the RNA would yield a fragment of longer length for most primer pairs. All reverse transcription reactions were tested for DNA contamination using primers that generate fragments spanning introns. (e.g. β-actin).

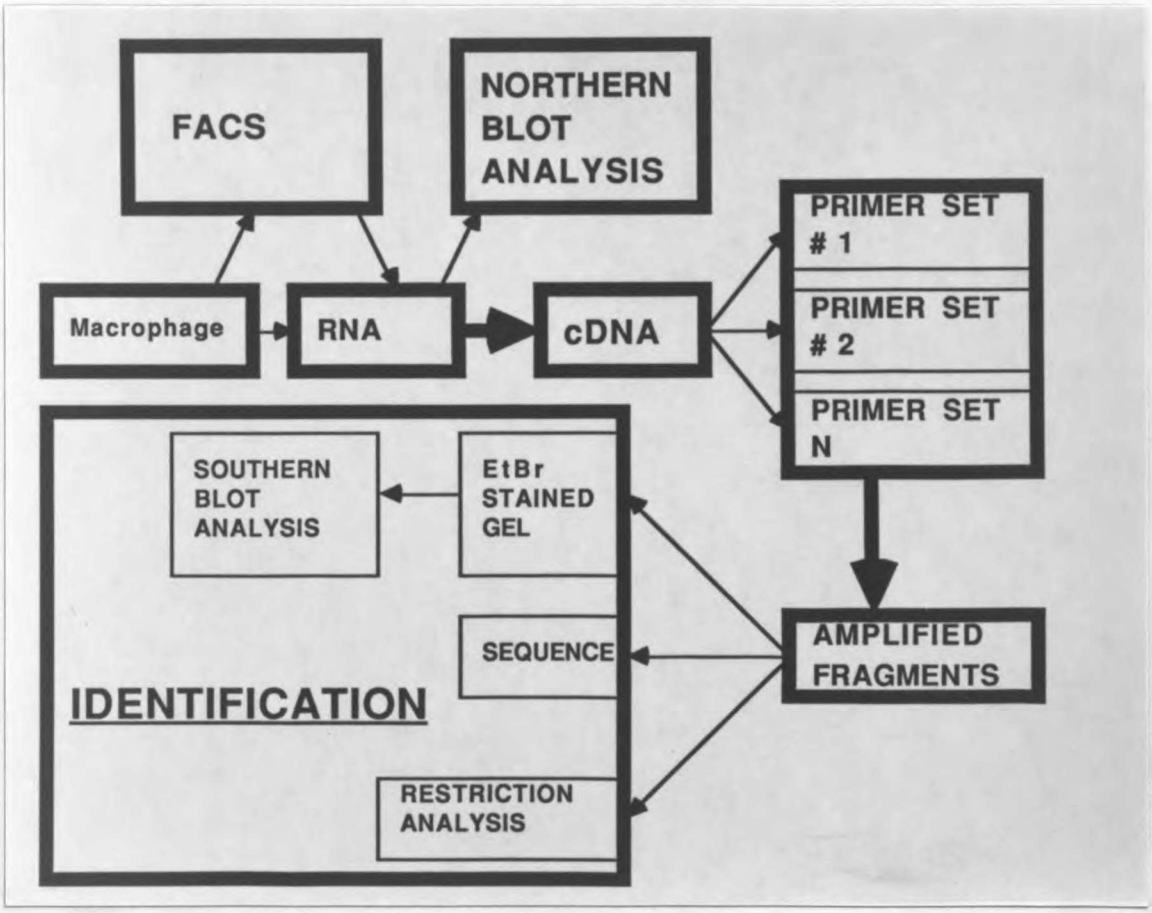
FACS
NORTHERN BLOT ANALYSIS
Macrophage
RNA
cDNA
PRIMER SET # 1
PRIMER SET # 2
PRIMER SET N
SOUTHERN BLOT ANALYSIS
EtBr STAINED GEL
SEQUENCE
AMPLIFIED FRAGMENTS
IDENTIFICATION
RESTRICTION ANALYSIS

Figure 1a

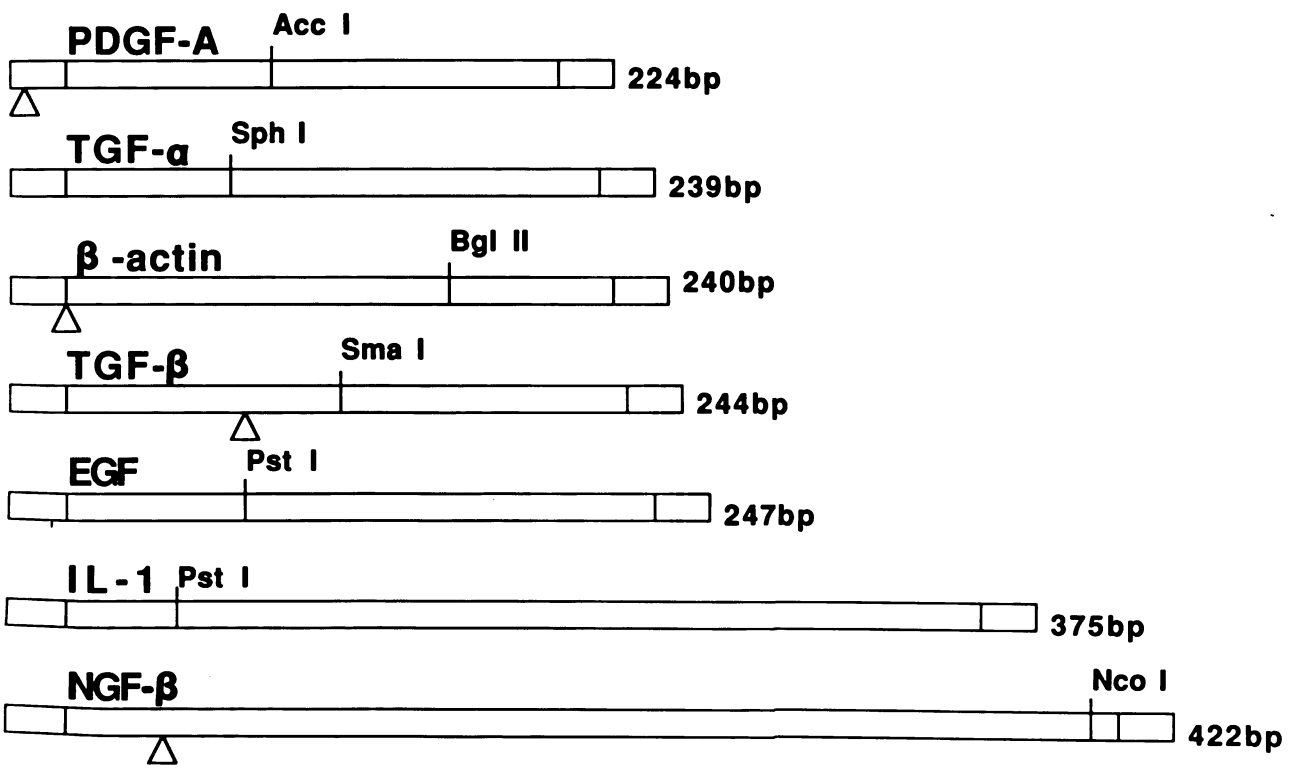
PDGF-A
Acc I
224bp
TGF-α
Sph I
239bp
β -actin
Bgl II
240bp
TGF-β
Sma I
244bp
EGF
Pst I
247bp
IL-1
Pst I
375bp
NGF-β
Nco I
422bp

Figure 1b

Table 1. Oligonucleotides used in RNA phenotyping amplification. Sequences are from published sources (*13*).

Oligonucleotide	Sequence (5′-3′)	Location in nucleotide sequence
β-actin 5′	GTGGGCCGCTCTAGGCACCA	25-44
β-actin 3′	TGGCCTTAGGGTGCAGGGGG	269-245
TGF-α 5′	ACCTGCAGGTTTTTGGTGCAG	58-78
TGF-α 3′	GCAGACGAGGGCACGGCACCA	297-277
TGF-β 5′	AAGTGGATCCACGAGCCCAA	1277-1296
TGF-β 3′	GCTGCACTTGCAGGAGCGCA	1521-1502
PDGF-A 5′	CCCCTGCCCATTCGGAGGAAGAGA	622-645
PDGF-A 3′	CTTGGCCACCTTGACGCTGCGGTG	848-826
EGF 5′	CCAGTTCAGTAGAAACTGGG	3953-3972
EGF 3′	TGGTTTCTAATGATTTTCTCC	4200-4181
NGF-β 5′	CCAAGGACGCAGCTTTCTAT	248-267
NGF-β 3′	CTCCGGTGAGTCCTGTTGAA	649-630
IL-1α 5′	AGATTCTGAAGAAGAGACGG	305-324
IL-1α 3′	AGCAACACGGGCTGGTCTTC	680-661
IGF-1A 5′	GGACCAGAGACCCTTTGCGGGG	217-239
IGF-1A 3′	CCACTGAAGCCTACAAAAGCAGCC	416-440

Figure 2 (A) Fragments of predicted size. Fragments generated from cDNA were generated from known sources of mRNAs (*3,4,13*) by each sequence-specific primer pair. RNA was isolated from: mouse brain for TGF-α; thioglycollate-elicited peritoneal macrophages (TEPM) stimulated with LPS for 3 hours for IL-1α; BALB/c 3T3 fibroblasts for NGF-β; and male mouse submaxillary glands for EGF. Whole RNA (0.1 μg) was reverse-transcribed by incubation with 100 U of MMLV-RT and 0.2 μg oligo-dT as primer for 1 hour at 37 ° C. One tenth of the resulting cDNA was amplified with 1 U of *Thermus aquaticus* (Taq) polymerase (Perkin-Elmer Cetus) and 1 μM primers during 60 cycles of PCR. Each cycle included denaturation at 93°C, reannealing of primer and fragment at 55°C, and primer extension at 72°C. Six microliters of 50 μl PCR reaction mix were fractionated electrophoretically in a 3% NuSieve GTG/1% SeaKem ME agarose gel. Gels were run at 80 V until the bromphenol blue dye front had migrated 6 cm and were then stained with ethidium bromide. Arrows indicate sizes of a Hae III digest of ϕX174 in the marker lane (M). Each band is the size predicted for the amplified cDNA (see Fig. 1B). Lack of a second higher band indicates that the RNA was not contaminated with genomic DNA. (B) Restriction enzyme analysis of DNA fragments generated from RT-PCR. Forty microliters of ethanol-precipitated RT-PCR fragments were digested with the restriction enzymes shown in Fig. 1B, according to the manufacturers' instructions (Bethesda Research Labs and Promega Biotech). Paired digested (C) and undigested (U) fragments were fractionated by electrophoresis on a 4% agarose gel and stained with ethidium bromide. Each digested PCR fragment was obtained from cDNA synthesized from macrophage mRNA. The diagnostic fragments are β-actin, 151 bp; IL-1α, 267 bp; TGF-β, 125+119 bp; TGF-α, 159 bp; PDGF-A, 129 bp. Size markers (M) are as indicated in Fig. 2A. Incomplete restriction cuts may de due to loss of the restriction site via inaccurate reading by Taq polymerase during early PCR cycles. (C) Resolution of 3-fold differences in input cRNA by RT-PCR. cRNA was produced by T7 polymerase from a pBR322/IL-1 con-

struct plasmid (unpublished results, D. Mark) as template, purified by oligo-dT chromatography, and quantified by absorbance at 260 nm. Serial 3-fold dilutions of cRNA were added to 1 μg of carrier RNA, so that the RT mixture contained 3 x 10^4 to 1.3 x 10^7 copies of cRNA. After RT, the cDNA was amplified by 40 cycles of PCR in the presence of [^{32}P]dCTP to follow incorporation into the specific cDNA fragment band. After separation on agarose gels, bands were excised and radioactivity was determined. (D) Generation of a β-actin signal by RT-PCR from a single macrophage. RNA, isolated as described in Fig. 3A, from 1 (■), 10^2 (■), and 10^4 (■) TEPM was reverse-transcribed, and the cDNA was amplified by PCR. PCR products sampled at 35-85 cycles were electrophoresed in 4% agarose gels, stained with ethidium bromide, photographed, and scanned with a Biorad densitometer. Peak areas are given as relative units.

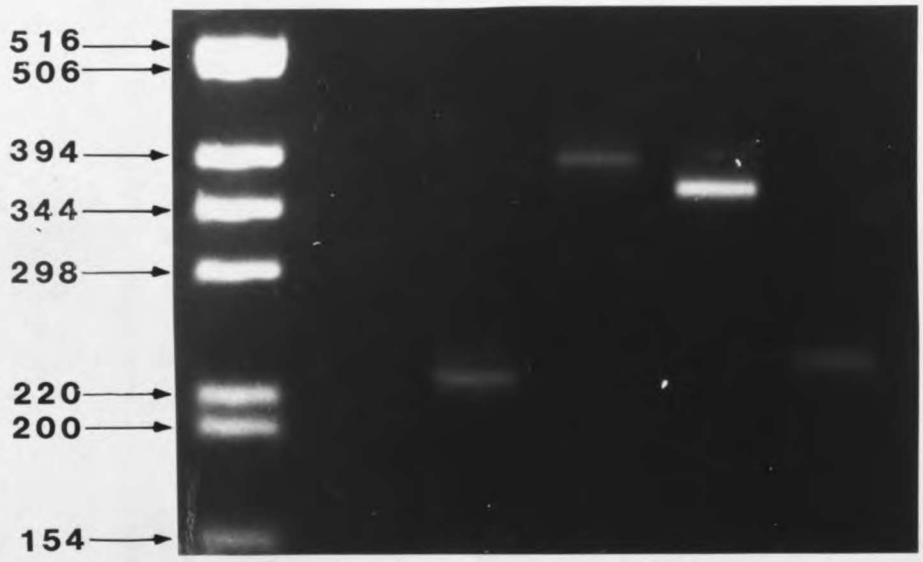

Figure 2a

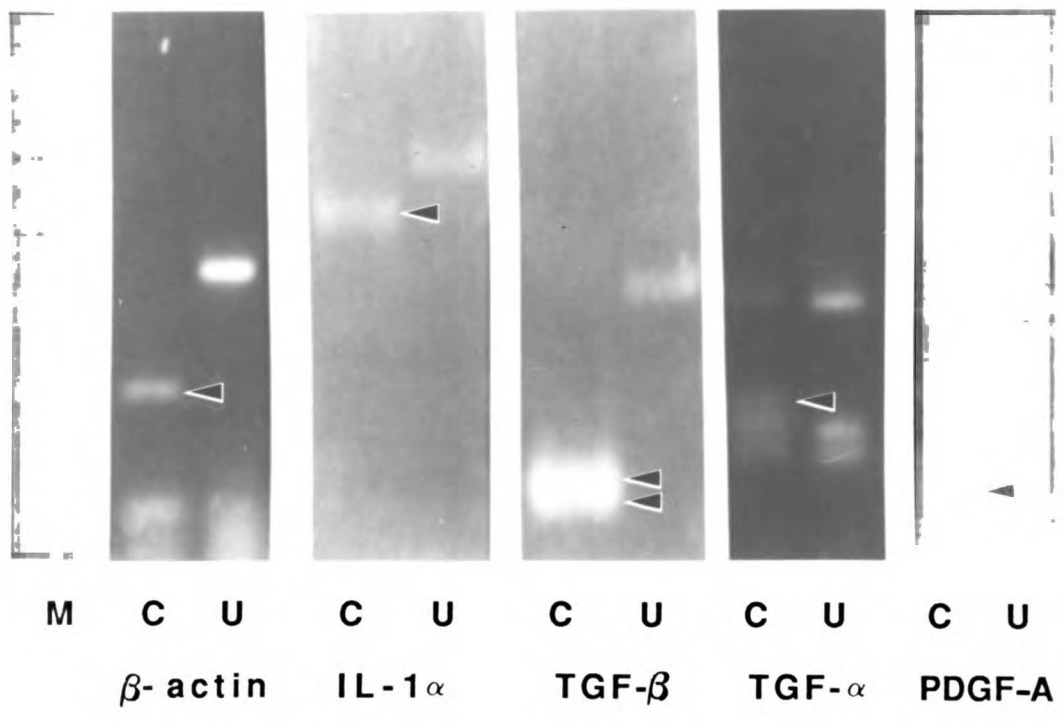

Figure 2b

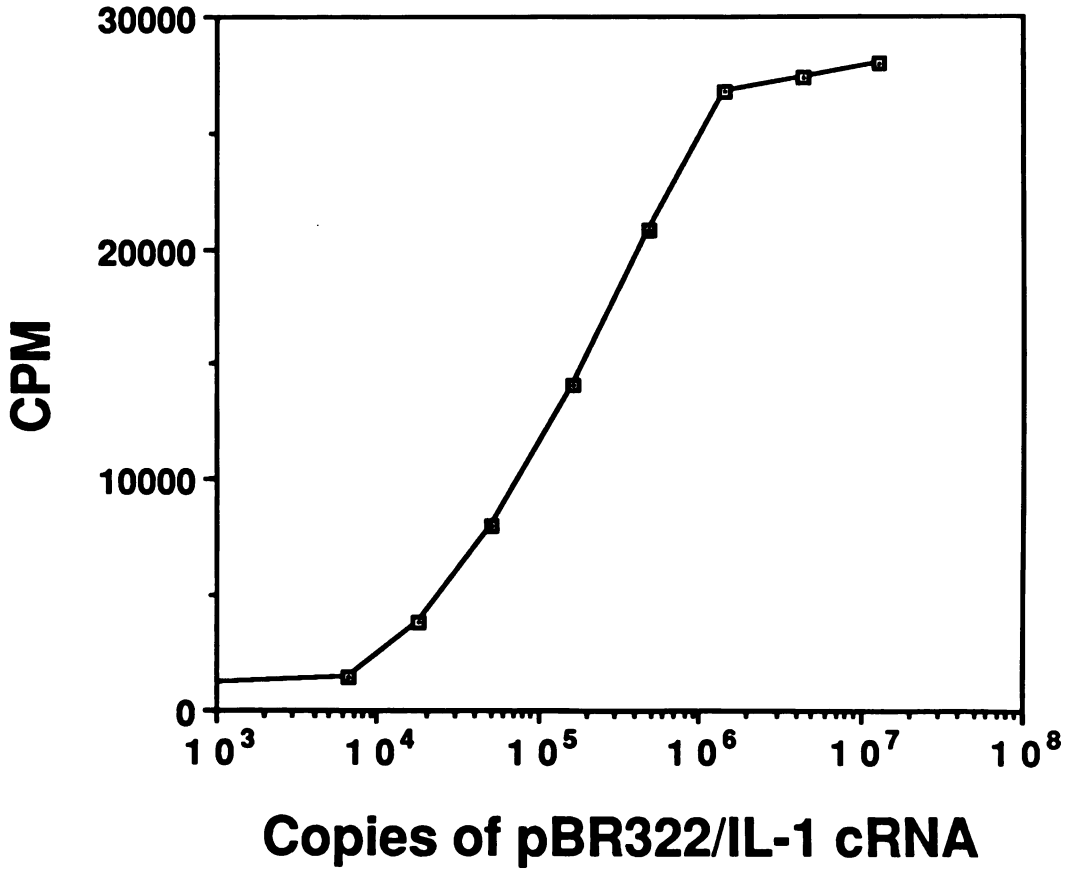
30000
20000
10000
0
CPM
10^3
10^4
10^5
10^6
10^7
10^8
Copies of pBR322/IL-1 cRNA

Figure 2c

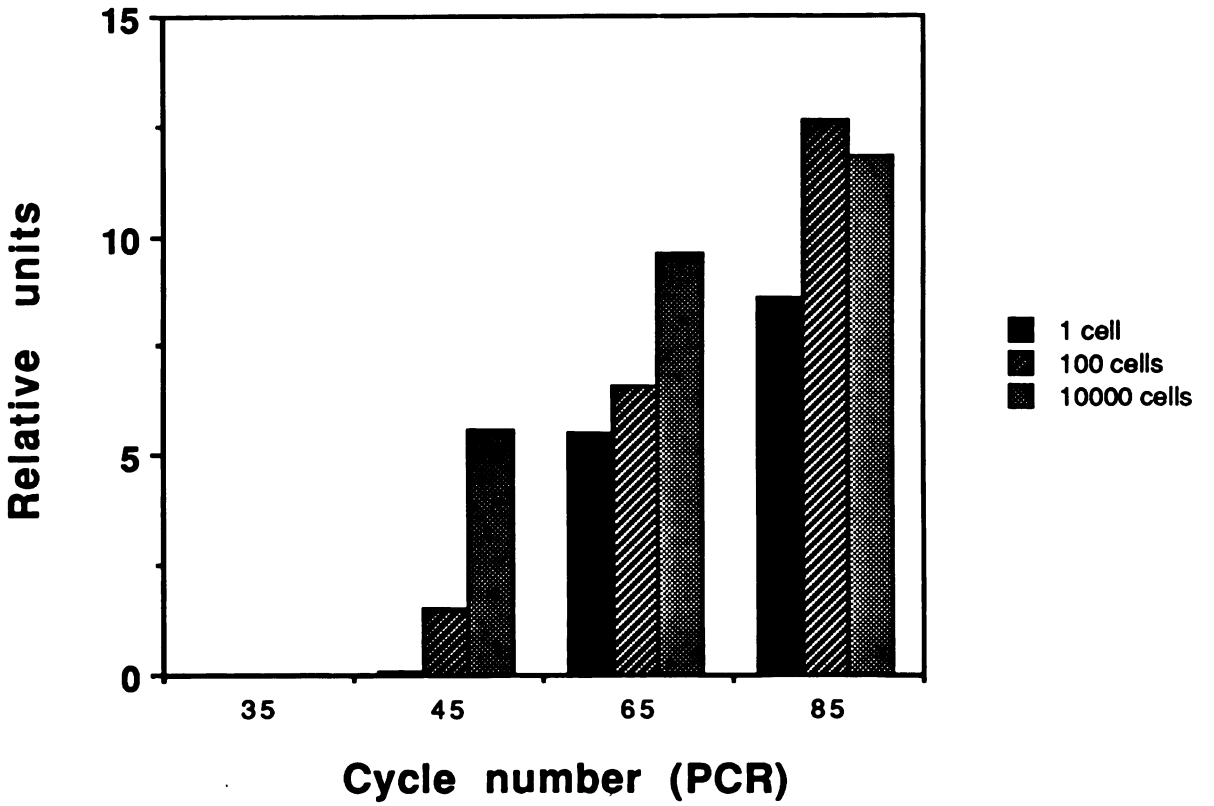
15
10
5
0
Relative units
35
45
65
85
Cycle number (PCR)
1 cell
100 cells
10000 cells

Figure 2d

Figure 3 (A) Expression of TGF-α mRNA transcripts by adherent cells isolated from wound cylinders. Stainless steel wound cylinders (*9*) were implanted in the flanks of mice (CD1, Charles River Laboratories). After 6 days cells and wound fluid were aspirated from the cylinders in the presence of acid-citrate-dextrose anticoagulant. Cells from 8 cylinders were incubated on a glass coverslip for 1 hour, and adherent cells (~ 10^3) were purified by extensive washing with 0.9% NaCl. GuSCN solution (100 μl) containing 10 μg of *Escherichia coli* rRNA as carrier was added to the adherent cells. The mixture was overlaid onto 100 μl of 5.7 M CsCl, and RNA was isolated after centrifugation for 20 x 10^6 *g*-min/cm in a TLA 100 rotor of a Beckman TL-100 centrifuge. The yield of RNA was usually 35-50%. RNA was reverse-transcribed, and cDNA fragments were amplified as described in Fig. 2A. Size markers (M) are as indicated in Fig. 2A. Experiments were performed at least three times with independent cell preparations. (B) Expression of macrophage-specific F4/80 antigen and TGF-α antigen by adherent cells from wound cylinders. Glass-adherent wound cells were fixed and stained (22) with (**a-d**) F4/80; (**e,f**) nonimmune rat IgG; (**g-j**) anti-TGF-α polyclonal antiserum; or (**k,l**) normal rabbit serum. Arrows indicate negative cells. No signal was seen in cells stained with anti-TGF-α absorbed with 1,000-fold excess of synthetic TGF-α polypeptide (data not shown).

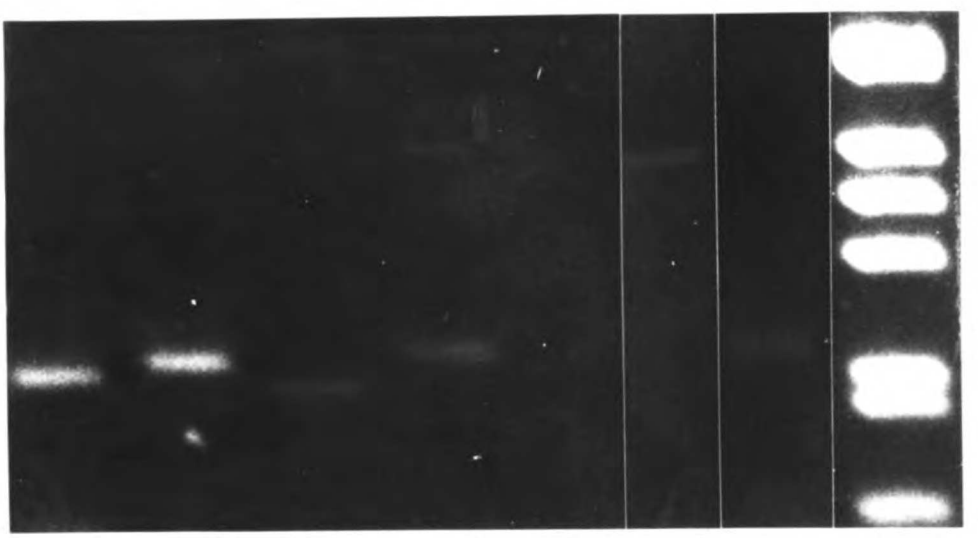

Figure 3a

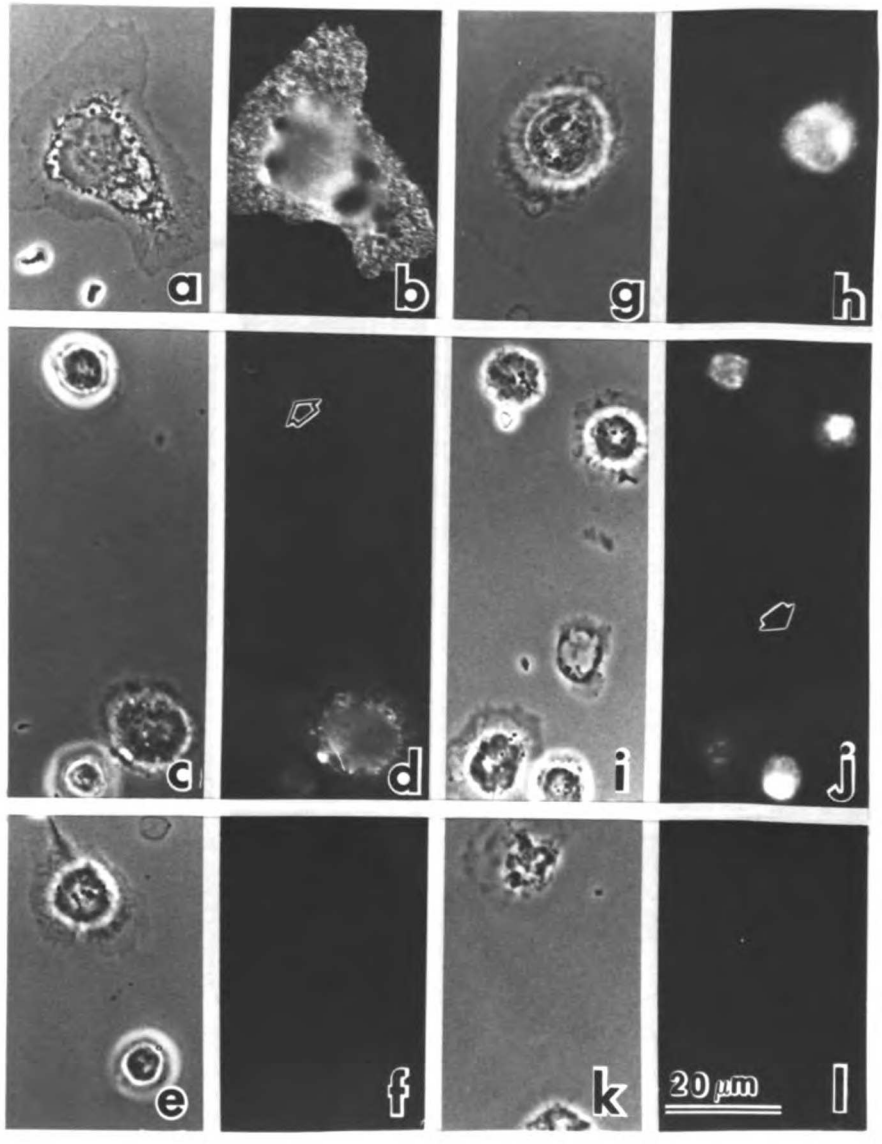

Figure 3b

for growth factor antigens. Half of the cells had macrophage morphology, and most of the rest were polymorphonuclear leukocytes. The macrophages had TGF-α immunofluorescence signals in the absence, but not the presence, of soluble competing TGF-α (Fig. 3B). Similar adherent cells were positive for the macrophage-specific antigen F4/80 (Fig. 3B). About half of the wound cylinder cells with macrophage features were also positive for PDGF antigen by immunofluorescence (data not shown). Wound fluid from the cylinders contained about 51 ng/l of TGF-α antigen, as assayed by the enzyme-linked immunoabsorbent assay (ELISA) described in Fig. 4E. Rabbit wound fluid contained 75 ng/l TGF-α/EGF, as measured by a competitive radioreceptor assay (Biomedical Technology, Inc., data not shown). These data provide evidence that adherent cells from wounds express TGF-α mRNA and that F4/80-positive wound macrophages express TGF-α antigen.

Because we observed EGF mRNA in adherent wound cells (Fig. 3A) but not in cultured or cell line macrophages, it was probable that nonmacrophage wound cells were transcribing EGF. To provide direct evidence for TGF-α transcription by macrophages, we next examined highly purified cultured macrophages and macrophage cell lines. Thioglycollate-elicited mouse peritoneal macrophages were stimulated with LPS (*11*) for 6 hours or incubated in control medium, then purified by fluorescence-activated cell sorting (FACS) using the F4/80 monoclonal antibody to select only macrophages. The RNA phenotype was assayed by the RT-PCR method. TGF-α was expressed only by LPS-stimulated macrophages (Fig. 4A), whereas TGF-β was expressed constitutively, as previously reported (*4*). LPS stimulated, cultured macrophages expressed IL-1α, G-CSF, GM-CSF, M-CSF, basic FGF, IGF-1, and PDGF-A but did not express nerve growth factor-β (NGF-β) or EGF transcripts (data not shown). Therefore, FACS-purified macrophages were free of contaminating fibroblasts, which express NGF-β (*12*).

Mouse macrophage cell lines stimulated with LPS also expressed TGF-α mRNA (Fig.

Figure 4 (A) Expression of TGF-α mRNA transcripts by cultured macrophages stimulated with LPS. Adherent thioglycollate-elicited peritoneal exudate cells (*11*) were cultured for 6 hours in the presence of LPS (10 μg/ml) or in medium alone (control). Macrophages were selected by FACS as the brigtest 10% of cells binding F4/80. RNA from these F4/80-positive cells was reverse-transcribed, and cDNA fragments specific for TGF-α and TGF-β were amplified as described in Fig. 2A. Each lane corresponds to 1,000 cells. (B) Expression of TGF-α mRNA transcripts by 4 of 5 mouse macrophage cell lines stimulated with LPS. Cells from cell lines described in ref. 23 were grown to confluence on plastic and stimulated for 6 hours with LPS (10 μg/ml). RNA was isolated, and cDNA fragments for TGF-α and TGF-β were generated as described in Fig. 2A. Each lane corrspondds to 2,000 macrophages. PCR in A and B was terminated after 60 cycles. (C) Expression of TGF-α mRNA transcripts by P388D1 macrophages stimulated with LPS. RNA (20 μg) from LPS-stimulated or control P388D1 cells was fractionated on a 1% denaturing agarose gel, transferred to a nylon membrane, and UV-cross-linked (*24*). The blot was hybridized with a random-primed plasmid insert from mouse TGF-α cDNA (a gift of R. Derynck) and then rehybridized with a random-primed β-actin plasmid (a gift of M. Kirschner). Arrows indicate migration of 28S and 18S rRNA bands. (D) Expression of TGF-α mRNA transcripts by lipid-loaded or LPS-stimulated cultured macrophages in a time-dependent manner. TEPM were cultured in serum-free medium (control) or stimulated with LPS (10 μg/ml) or AcLDL (50 μg/ml, Biomedical Technology, Inc.) for 3-48 hours. cDNA fragments were generated as described in Fig. 2A. * A similar pattern of TGF-α expression was seen in TEPM treated for 3 or 6 or 9 hours with LPS (data not shown). (E) *a.* Expression of TGF-α antigen by LPS-stimulated cultured macrophages in a time-dependent manner. TEPM were cultured in serum-free medium alone (●) or with LPS (□). Medium was collected at 6, 12, 24, and 48 hours, dialyzed against 0.2 M acetate buffer, pH 4.5, and resuspended at 10X concentration, then

assayed in a competitive ELISA (*25*). Error bars indicate mean ± SE (n=6). As much as 10 ng of EGF (Collaborative Research) was negative in the assay. Plus sign (+) indicates TGF-α in mouse wound fluid. (E) *b.* Biosynthesis of authentic TGF-α polypeptide by LPS-stimulated cultured macrophages. LPS-stimulated TEPM were radiolabeled with [^{35}S]cysteine (100 μCi/ml; spec. act., 1135 Ci/mmol) for 24 hours. Conditioned medium was processed as described in Fig. 4E(a) and was fractionated on a TSK 4000SW (30 cm)-3000SW (60 cm) size exclusion column system equilibrated and run with a mobile phase of 0.1% trifluoroacetic acid - 40% acetonitrile. The 3-20 kDa fractions were immunoprecipitated with anti-TGF-α antiserum (i) or nonimmune rabbit serum (ii), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7-15% gradient gel under denaturing conditions, and then autoradiographed. The immunoprecipitated, biosynthetically labeled band migrated slightly higher than the 6 kDa standard but at the same size as ^{125} I-labeled synthetic TGF-α (Biotope), which was used as a control (not shown). (F) Expression of intracellular TGF-α antigen by LPS-stimulated cultured macrophages. TEPM were stimulated for 24 hours with 10 μg/ml LPS or were cultured in serum-free medium, then fixed and stained (*13*). (a,b) Anti-TGF-α staining of LPS-stimulated TEPM; open arrows indicate perinuclear staining concentrated in the Golgi region; (c,d) anti-TGF-α staining of unstimulated TEPM; (e) staining of LPS-stimulated TEPM with with nonimmune rabbit serum; (f) Staining of LPS-stimulated TEPM with anti-TGF-α absorbed with a 1,000-fold excess of TGF-α polypeptide; arrows indicate diminished perinuclear staining compared to (b). (a,c) Phase-contrast micrographs; (b,d,e,f) immunofluorescence. (G) Secretion of mitogenic activity by LPS-stimulated elicited macrophages and P388D1 cells. (a) P388D1 and (b) TEPM were cultured with LPS (10 μg/ml) for 48 hours. Conditioned medium was collected and processed as described in Fig. 4(E). Ten milliliters of 10X concentrate in phosphate-buffered saline (PBS) was applied to a heparin-Sepharose column (Pharmacia) and eluted with a continuous gradient of 0-3.3 M

NaCl in PBS. Column fractions were dialyzed against PBS and assayed for mitogenic stimulation of BALB/c 3T3 cells (*26*). The dashed lines indicate the mitogenic stimulation of the positive control (10% fetal calf serum), and the solid lines indicate the mitogenic stimulation of the negative control (10% RPMI 1640). Pooled mitogenic peaks numbered 2, 3, 5, and 6 were assayed for specific growth factors (see Table 2).

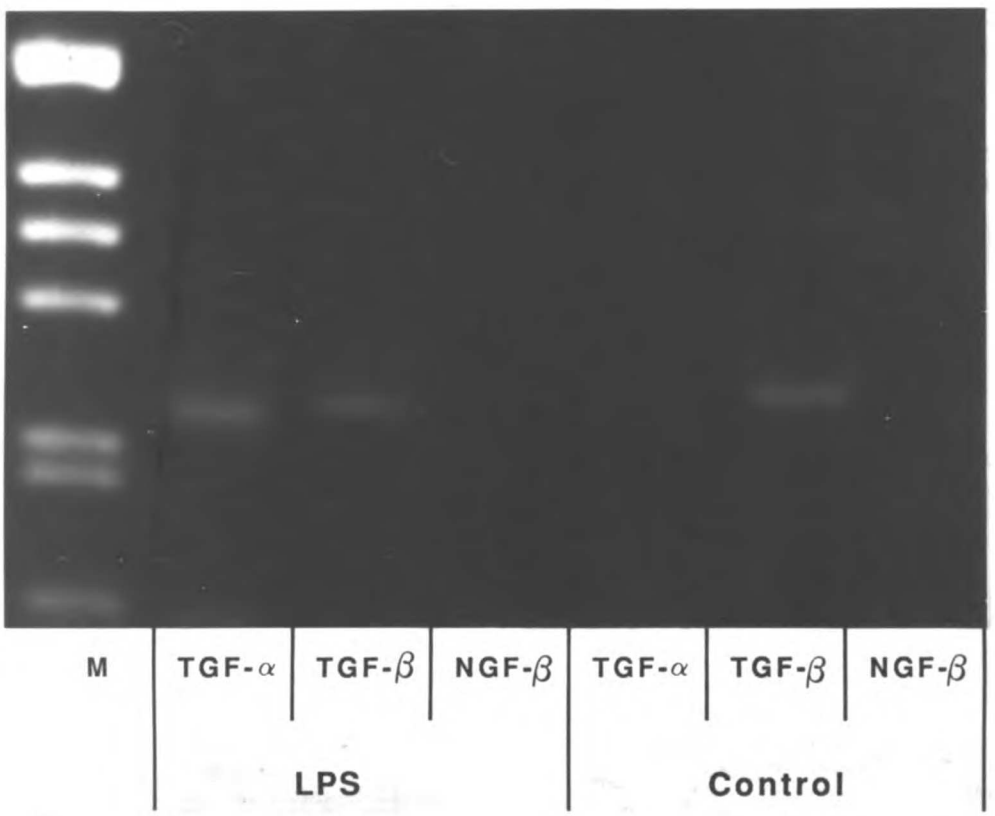

Figure 4a

4B). The number of TGF-α transcripts in the P388D1 macrophage cell line was increased by treatment with LPS, and their single ~ 4.5 kb size, determined by RNA blot analysis (Fig. 4C), matches the size of the rat brain TGF-α transcript (*13*). TGF-α transcripts were detected within 3 hours of treatment of elicited macrophages with LPS, or after lipid-loading macrophages with acetylated low-density lipoproteins (AcLDL), another stimulus relevant to tissue injury and wound healing (*14*) (Fig. 4D). Transcript levels remained high for 6 or 9 hours of LPS treatment, but had disappeared by 48 hours.

These data suggest that macrophages are capable of synthesizing TGF-α mRNA transcripts under relevant circumstances. But transcription of growth factors does not always correspond qualitatively or quantitatively to translation (*4*). We next asked whether macrophages in culture translate the TGF-α mRNA. TGF-α antigen was secreted by LPS-stimulated elicited macrophages in a time-dependent manner, as determined by ELISA, at concentrations similar to those found in wound fluid (Fig. 4E). The antigen detected in the ELISA was an authentic macrophage translation product, as demonstrated by immunoprecipitation of TGF-α of ~ 6 kDa from biosynthetically labeled secreted macrophage proteins (Fig. 4E). At least 90% of the LPS-stimulated cultured macrophages actively synthesized TGF-α, as analyzed by immunocytochemistry, whereas few, if any, of the unstimulated cultured macrophages expressed intracellular TGF-α (Fig. 4F). Cultured P388D1 macrophages or elicited peritoneal macrophages were next stimulated with LPS for 48 hours in serum-free medium to determine production of mitogenic activity. Medium was concentrated by dialysis and lyophilization, fractionated by heparin-Sepharose affinity chromatography, and assayed for mitogenic stimulation of quiescent confluent BALB/c 3T3 fibroblasts (Fig. 4G). As described by Shing *et al.* (15), EGF does not bind to heparin-Separose, PDGF binds and elutes at low NaCl concentration, and basic FGF binds and elutes at high NaCl concentration. Mitogenic activity produced by P388D1 cells eluted under these

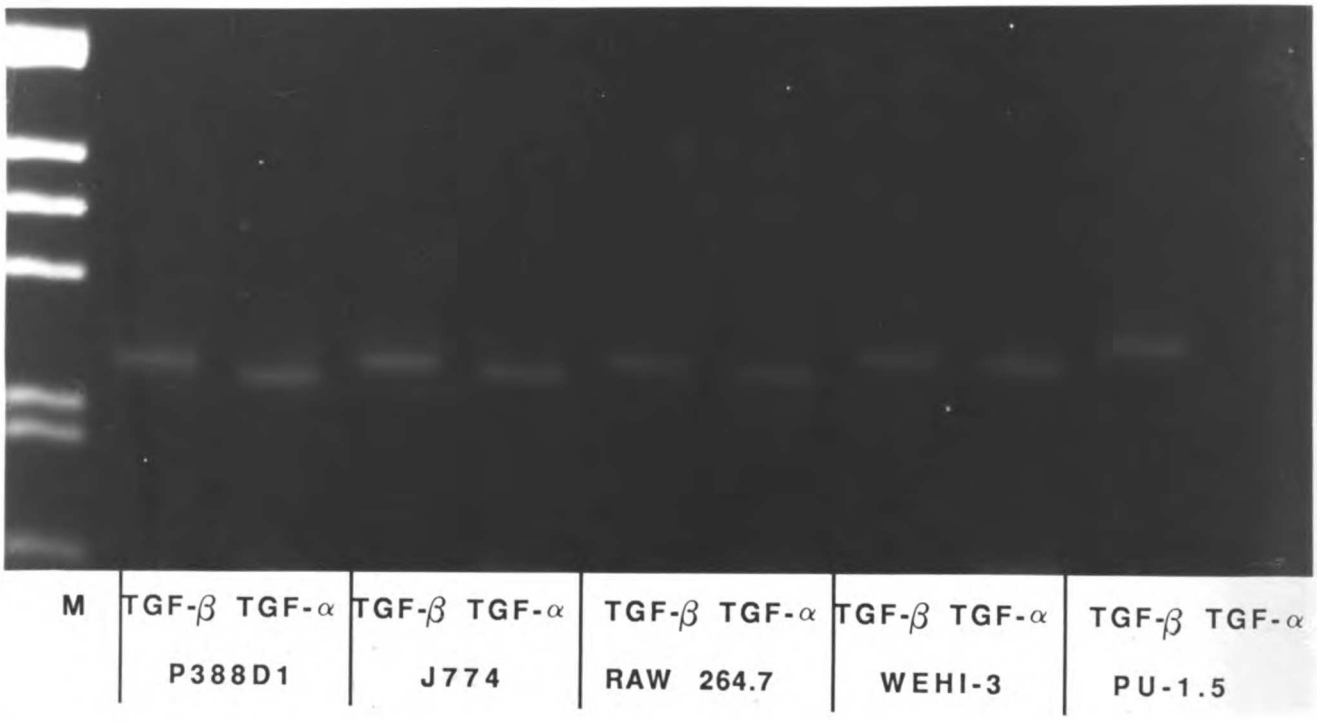
M
TGF-β TGF-α
P388D1
TGF-β TGF-α
J774
TGF-β TGF-α
RAW 264.7
TGF-β TGF-α
WEHI-3
TGF-β TGF-α
PU-1.5

Figure 4b

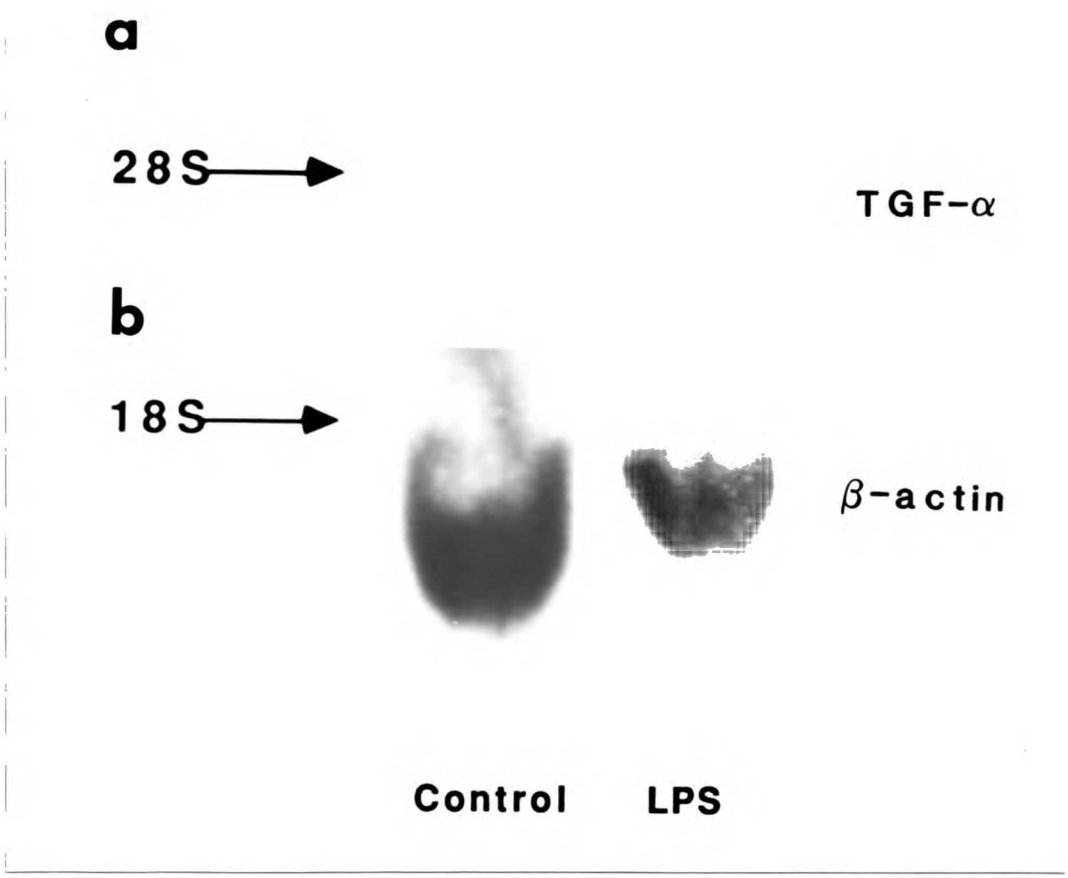

Figure 4c

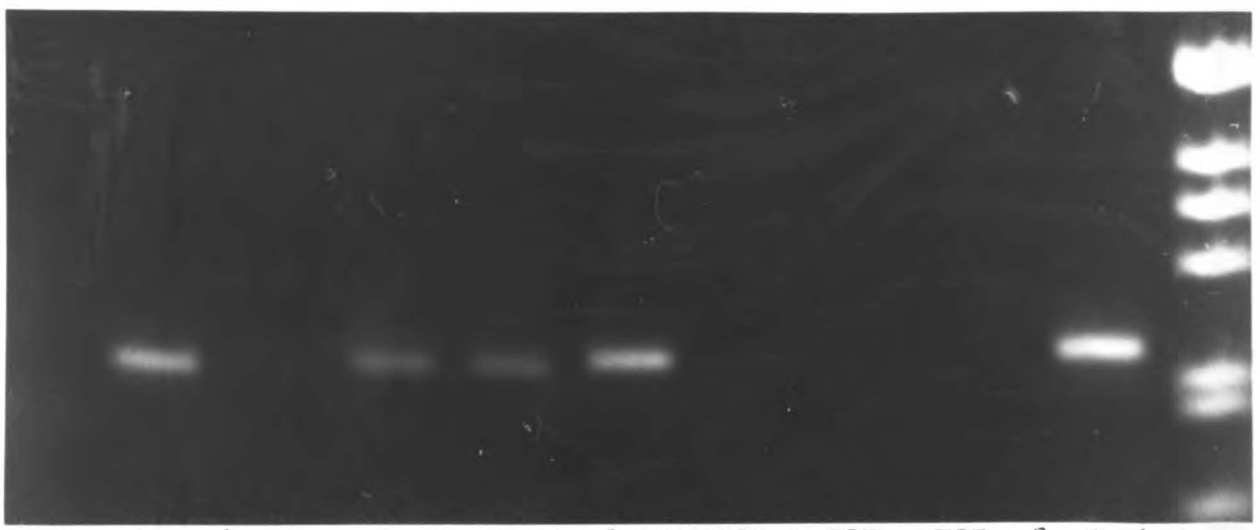
TGF-α Control
β-actin Control
TGF-α Control
TGF-α AcLDL
TGF-α LPS
β-actin Control
TGF-α Control
TGF-α AcLDL
TGF-α LPS
β-actin Control
M
Time (hr) 0
3
48

Figure 4d

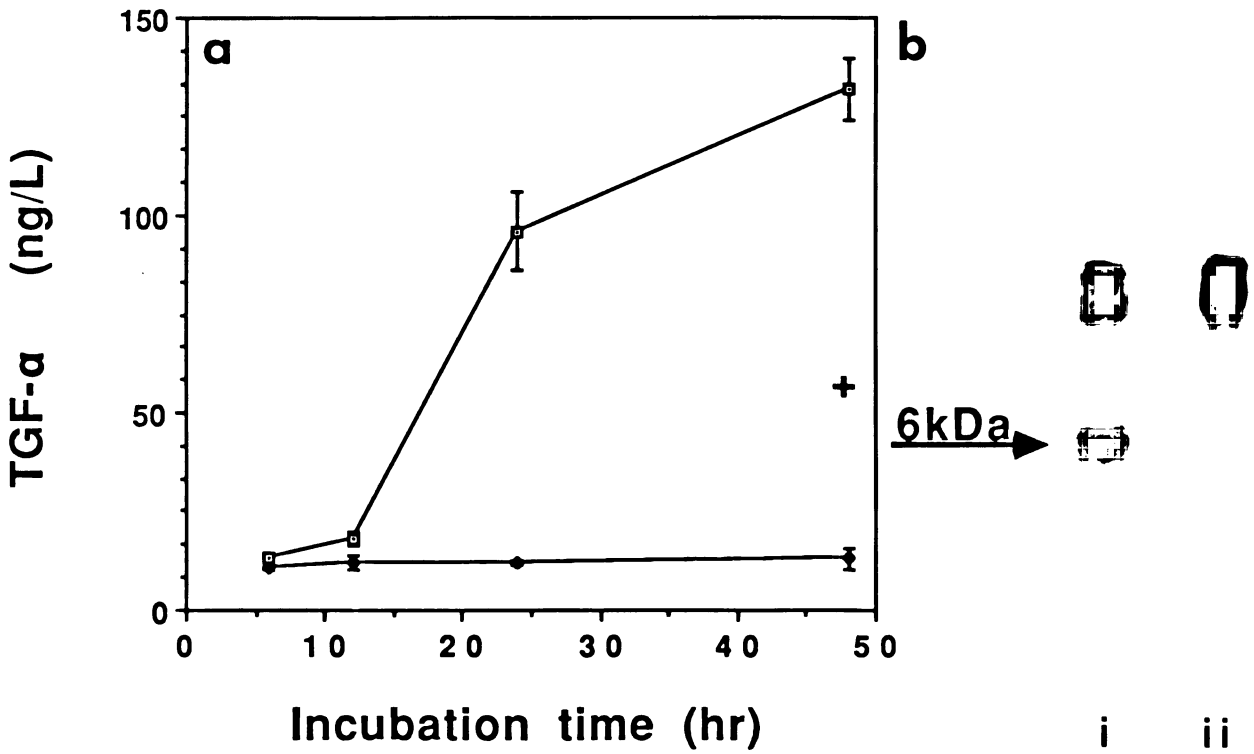
a
b
150
100
50
0
TGF-α (ng/L)
0
10
20
30
40
50
Incubation time (hr)
+
6kDa
i
ii

Figure 4e

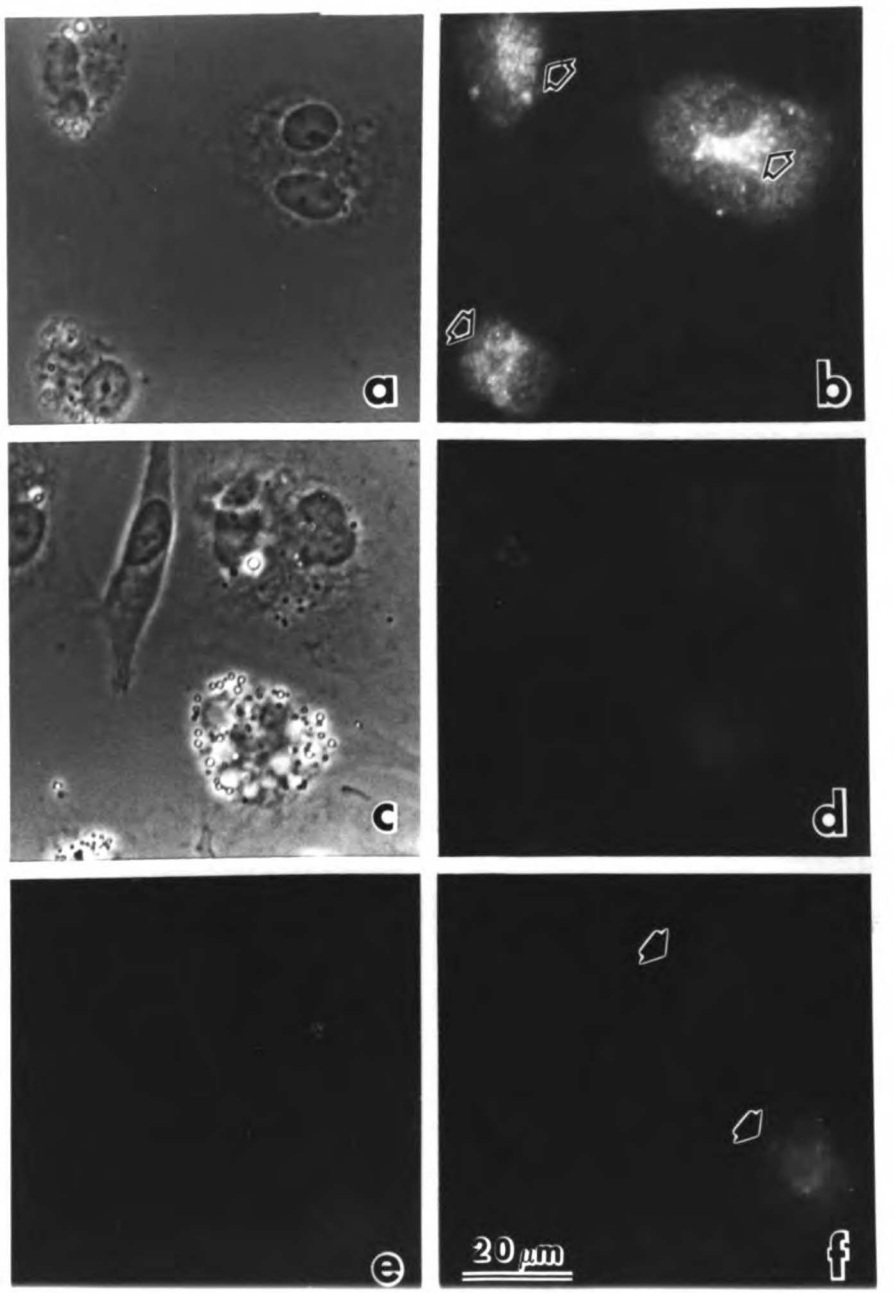

Figure 4f

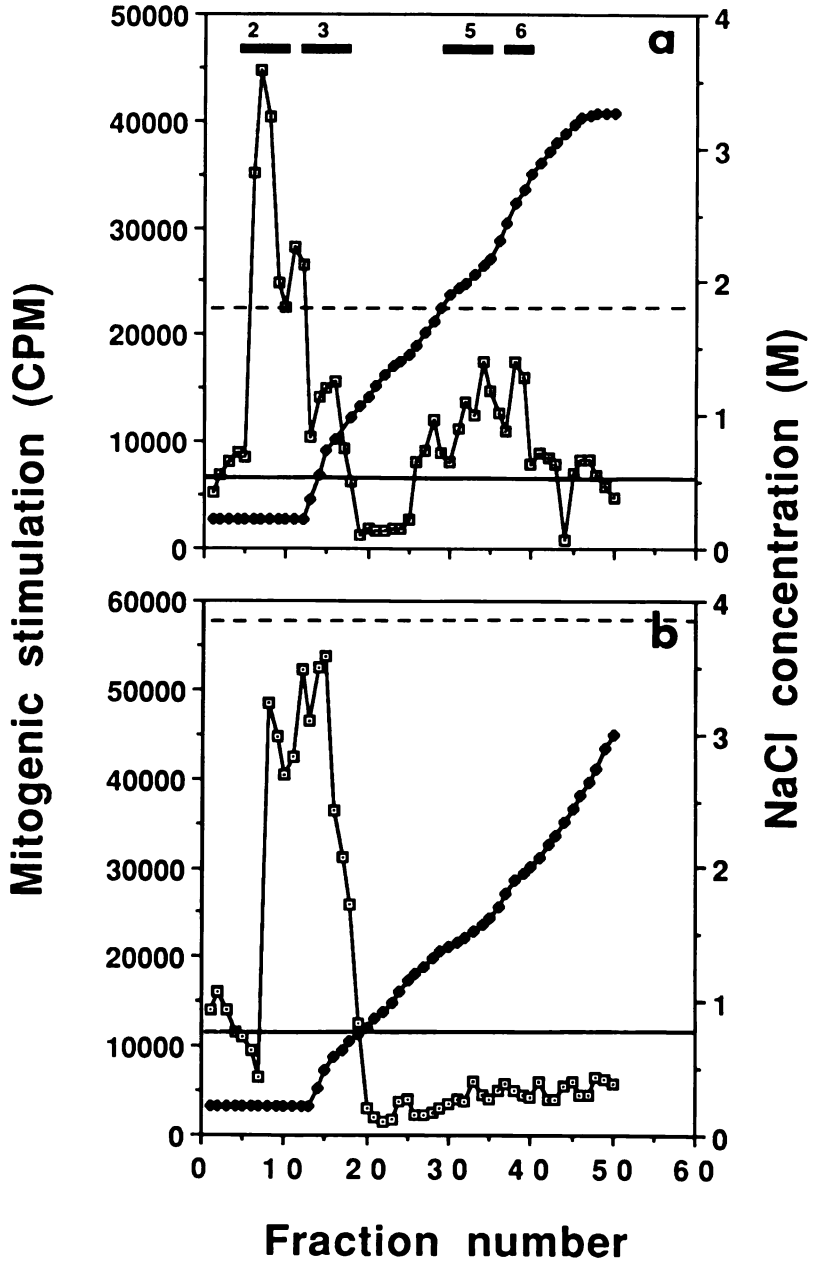
Mitogenic stimulation (CPM)
NaCl concentration (M)
Fraction number
a
b
2
3
5
6
50000
40000
30000
20000
10000
0
60000
0
10
20
30
40
50
60
4
3
2
1

Figure 4g

three conditions. These peaks were pooled and assayed by radioreceptor assay to detect TGF-α/EGF or by dot immunoblot assay to detect PDGF, TGF-β, or basic FGF (Table 2). TGF-α was a major component of the total mitogenic protein and activity in macrophage-conditioned medium (Fig. 4E; Table 2). Similar elution profiles were seen for P388D1 cells and elicited macrophages, except that FGF was not secreted by elicited macrophages. We do not know if the FGF in conditioned medium of P388D1 cells was due to secretion or to a low incidence of cell death. The viability of both elicited peritoneal macrophages and P388D1 macrophages was greater than 98% in all cultures, but the intracellular pool of basic FGF in P388D1 was not quantified.

An essential process such as wound healing has redundant components. TGF-α is thought to mediate epidermal regrowth, angiogenesis, and formation of granulation tissue in vivo (*8*). Macrophage-derived TGF-β and basic FGF are potentially angiogenic. Macrophage-derived IL-1, TGF-β, PDGF, and basic FGF could cause formation of granulation tissue. TGF-β may mediate epidermal regrowth (*16*), but this is likely to be an indirect function of its powerful chemoattraction for macrophages (*17*). Taken together, our data indicate that TGF-α delivered by macrophages may be a direct mediator of wound healing. The discovery that wound macrophages express multiple growth factors, and produce them under wound healing conditions in vivo, will have important biological ramifications. The technology developed to detect transcripts from a single cell or a few cells should be applicable to many physiologic questions.

Table 2. Growth factors produced by LPS-stimulated P388D1 macrophages. P388D1 macrophages (1×10^8) were treated with 10 μg/ml LPS for 48 hours in serum-free medium. The conditioned medium (100 ml) was dialyzed and concentrated and then separated by heparin-Sepharose chromatography as described in Fig. 4G. Fractions eluting with the NaCl gradient were pooled as indicated in Fig. 4G(a) and assayed (18) for TGF-α/EGF by radioreceptor assay and for PDGF, TGF-β, and basic FGF by dot immunoassay The data, except for TGF-β, were from the same fractionation shown in Fig. 4G(a) and are expressed as amount per 100 ml of conditioned medium. TGF-β was measured in several different column runs, and the range of activities in relative units is shown. n.d., not determined.

Pool number	Eluting NaCl (M)	TGF-α/EGF (ng/100 ml)	FGF (ng/100 ml)	PDGF (ng/100 ml)	TGF-β (relative units)
2	0.2	31	0	0	0-5
3	0.4-0.7	0	0.1	25	0
4	1.1-1.4	n.d.	n.d.	n.d.	95-100
5	1.7-2.1	0	1.0	0	0
6	2.2-2.4	0	5.0	0	0

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18. The TGF-α/EGF radioreceptor assay was performed by using a kit from Biomedical Technology, Inc., according to the manufacturer's instructions. For the dot immunoblot assay [P.V. Talbot, R.L. Knobler, M.J. Buchmeier, *J. Immunol. Meth.* **73,** 177 (1984)] basic FGF column fraction samples or 1-10,000 pg basic FGF or acidic FGF (a gift of D.

Gospodarowicz) were dotted onto nitrocellulose, dried, and blocked with 3% normal rabbit serum, then with 1/1000 dilution of an anti-basic FGF monoclonal antibody (a gift of D. Gospodarowicz), developed with biotinylated rabbit anti-mouse IgG (Hyclone) diluted 1/1000, followed by alkaline phosphatase-labeled avidin (Vector) at 0.2 U/ml. For immunodot assays of PDGF, samples of macrophage-conditioned medium or 0.01-1.0 U PDGF (Collaborative Research) were dotted onto nitrocellulose, dried, blocked with 3% normal goat serum, then incubated with 1/500 dilution of goat anti-PDGF polyclonal antibody (a gift of G. Grotendorst) and developed with biotinylated sheep anti-goat IgG (Sigma) at 1/1,000, followed by alkaline phosphatase-labeled avidin (Vector), nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The TGF-β immunodot assay was performed in a similar manner using anti-TGF-β from R & D Systems, Inc.

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22. For immunofluorescence staining, adherent cells from wound cylinders or TEPM on 12-mm glass coverslips were fixed for 10-30 minutes in freshly prepared 2% paraformaldehyde, then washed in PBS containing 0.1% glycine. Cells to be stained with F4/80, a macrophage-specific rat monoclonal antibody (J.M. Austyn and S. Gordon, *Eur. J. Immunol.* **11,** 805 (1981) (a gift of S. Gordon, University of Oxford), were left unpermeabilized. Cells to be stained with anti-TGF-α (Peninsula Laboratories) were permeabilized with 0.25% Triton X-100 for 4 minutes. Nonspecific binding sites were then blocked with ovalbumin (1 mg/ml) for 30 minutes, followed by 5% skim milk powder for 30 minutes. Samples were then stained for

1 hour with F4/80 or nonimmune rat IgG at 20 μg/ml, or with anti-TGF-α or nonimmune rabbit serum at 1:100, diluted in 5% skim milk. After extensive washing, cells were then incubated with biotinylated $F(ab')_2$ fragments from either anti-rat IgG or anti-rabbit IgG (HyClone), diluted 1:100, washed, and stained with Texas Red-labeled streptavidin (Amersham). Cells were observed with a 63X water-phase PlanNeofluor lens on a Zeiss Photomicroscope III and photographed on Tri-X film rated at 800 ASA for 60-second exposures.

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26. Mitogenesis was assayed by determining [^{3}H]thymidine uptake into quiescent confluent BALB/c 3T3 fibroblasts. Fetal calf serum (10%) was used as a positive control, and 10% RPMI 1640 was used as a negative control for the mitogenesis assay by the methods of W.

Chapter 4

Studies on Other Macrophage-Derived Growth Factors.

Fibroblast Growth Factor

Ablation of macrophages precludes the proper healing of wounds (Leibovich et al., 1975). Regeneration of multiple cell types during wound healing must occur in order to replace the tissue lost in vivo. In vitro, these cell types divide when stimulated by growth factors. We set out to test the hypothesis that macrophages synthesize these growth factors when challenged by stimuli created during the healing process. At the start of the project, IL-1 was the only growth factor known to be both transcribed and translated by macrophages. No direct evidence of PDGF, bFGF, bombesin, erythropoietin, or colony stimulating activity expression in macrophage existed although a survey of the existing literature suggested that several uncharacterized growth factors could be produced by macrophages.

We began with the goal of characterizing these novel macrophage-derived growth factors by classical biochemical techniques. Four components are needed for this approach; 1) a source of growth factor: Secreted protens of P388D1 macrophages were used because IL-1 had been isolated from these macrophages and they have many characteristics of normal macrophages. 2) A bioassay for mitogenesis: Stimulation of ^{3}H-thymidine uptake by quiescent Balb/c 3T3 fibroblasts was used. 3) A method to check protein purity: Protein was identified by SDS-PAGE and silver staining after normalization of protein content. 4) A purification algorithm: Heparin-affinity chromatography was used because Shing *et al.* (1985) had just shown that EGF, PDGF, and FGF could be distinguished by their affinity for heparin.

In the mitogenic response assay (Fig. 1a,1b), confluent, mitotically quiescent Balb/c 3T3 fibroblasts were restimulated 7-14 days after plating with 10% (v/v) FCS in medium as a positive control and medium only as a negative control, as described in Chapter 3 or with 10% (v/v) of the dialyzed fractions from the heparin-Sepharose column. For affinity chromatography, Pharmacia C-10 columns containing 2 ml of Pharmacia heparin-Sepharose 6CL-B, were run at 4°C at 8 ml/hr maintained by an LKB Perpiplex pump. Chromatographic

separations were made with 100 ml of serum-free conditioned medium (CM) produced by culturing P388D1 cells at 10^6/ml, for 48 hr in the presence of 10 μg/ml of LPS. CM was dialyzed against 3 changes of 1 liter volumes of 0.1% acetate buffer, pH 4.5, ultrafiltered or lyophilized, and then reconstituted in 1/10 the volume in 0.15 M NaCl, 10 mM Tris-HCl buffer, pH 7.2. The 10 ml of the 10 x concentrate contained 2-5 mg of protein. All protein determinations were made by a Bradford (Bradford, 1976) assay. Half of the protein in the CM flowed through the heparin-Sepharose column and most of the rest eluted in a broad peak immediately upon starting the salt elution (0.15-0.6 M NaCl). Anionic heparin acts as a mild cation exchanger and most of the protein in this peak is thought to carry a positive charge (Shing, 1985). We found that P388D1 CM contained six mitogenic components separable by heparin-Sepharose chromatography: three peaks eluted during the flow through and washing of the column with loading buffer, one at a low salt concentration (0.3-0.6 M NaCl, pool 3), and two at strong heparin affinity (1.7-1.9 M NaCl), pool 5, and 2.1-2.3 M NaCl, pool 6, see Fig. 4g, Chapter 3). Silver stained SDS-PAGE gels (Fig. 2) indicate that pools 5 and 6 had together, approximately 25 ng of material at 14-18 kDa. This was determined by comparing by scanning densitometry with 50 ng and 100 ng of lysozyme run on the same gel (data not shown). The molecular weight range observed for the major proteins in pools 5 and 6 is similar to that seen for basic FGF and its immediate breakdown products (D. Gospodarowicz, personal communication). Moreover, bovine basic FGF elutes from similar columns at salt concentrations corresponding to pools 5 and 6 (data not shown). In support of the idea that MØ make bFGF, Baird et al. (1985) have demonstrated the presence of bFGF in cell lysates of elicited mouse peritoneal macrophages. In experiments, we took conditioned medium rather than cell lysates. It is interesting in this regard that bFGF contains no signal sequence (Abraham et al., 1986). Its mode of release from cells is unknown; it is possible that bFGF may be liberated only upon cell death. The P388D1 cell

Figure 1. Methods for generating quiescent Balb/c 3T3 and Swiss NIH 3T3 fibroblasts. (a) Balb/c3T3 fibroblasts were plated at 10,000 cells/well in 96 well microtiter plates (Corning) with the indicated amounts of fetal calf serum (% v/v) in the media and cell number reached a plateau at day 5. (b) Swiss NIH 3T3 fibroblasts were plated at 10,000 cells/well under similar contions as in (a) and reached plateau at day 7. Swiss fibroblasts were less consistent in serum response than Balb/c 3T3 fibroblasts and were not used in ^{3}H-thymidine uptake mitogenic assays. Balb/c 3T3 fibroblasts wree used only between passage 70 and 85. Quiescent Balb/c 3T3 fibroblasts that had been cultured in 10% fetal calf serum for 7-14 days were used to assay for mitogenesis by ^{3}H-thymidine. Control media or fetal calf serum or macrophage CM as added back at 10% v/v/ to quiescent cells, ^{3}H-thymidine (1 μCi, specific activity 25 Ci/mmol) was added 12 hr later and cells were aspirated onto glass filters and lysed (using a PhD cell harvester) 24 hr after addition of ^{3}H thymidine.

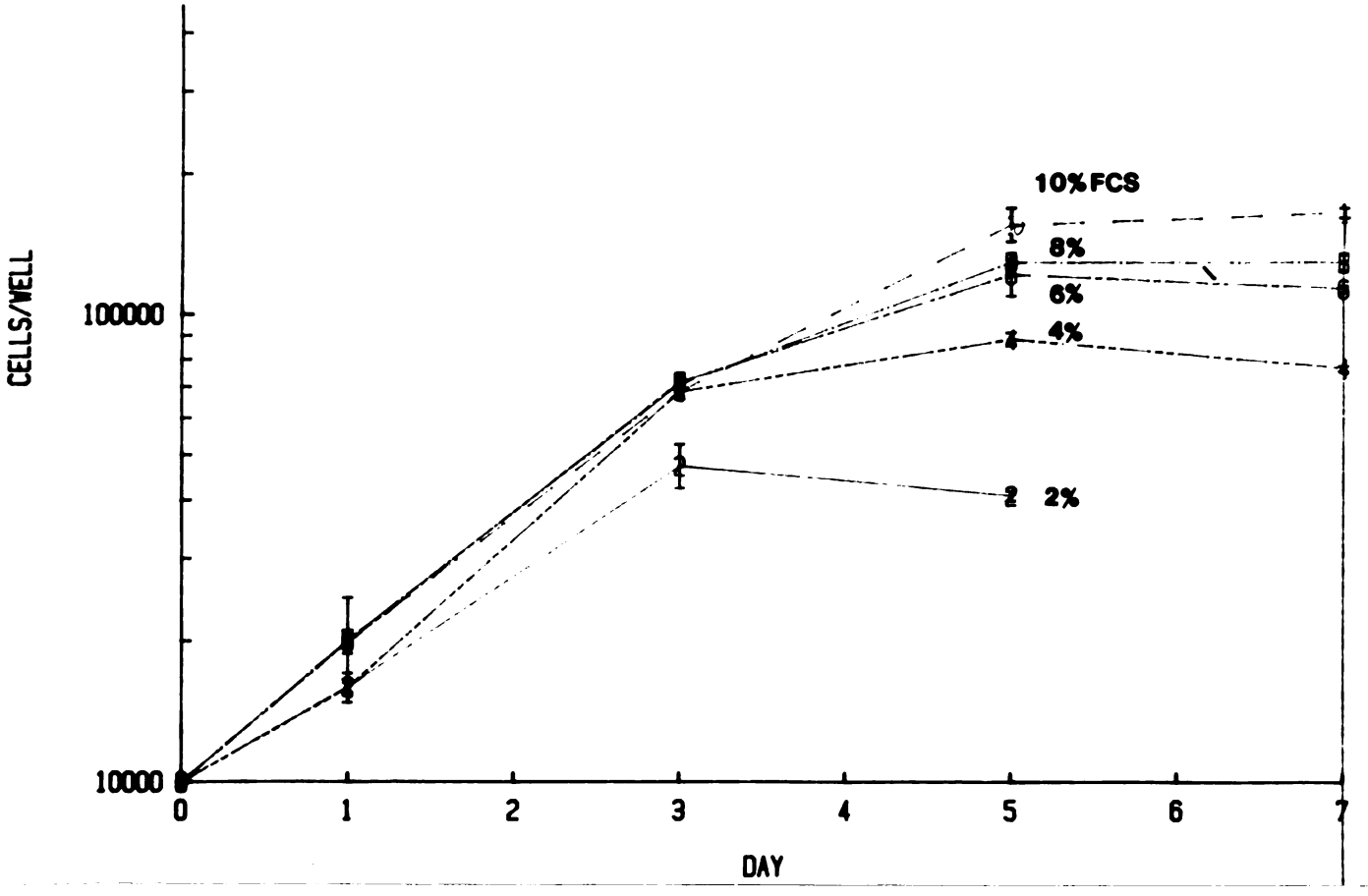


Figure 1a

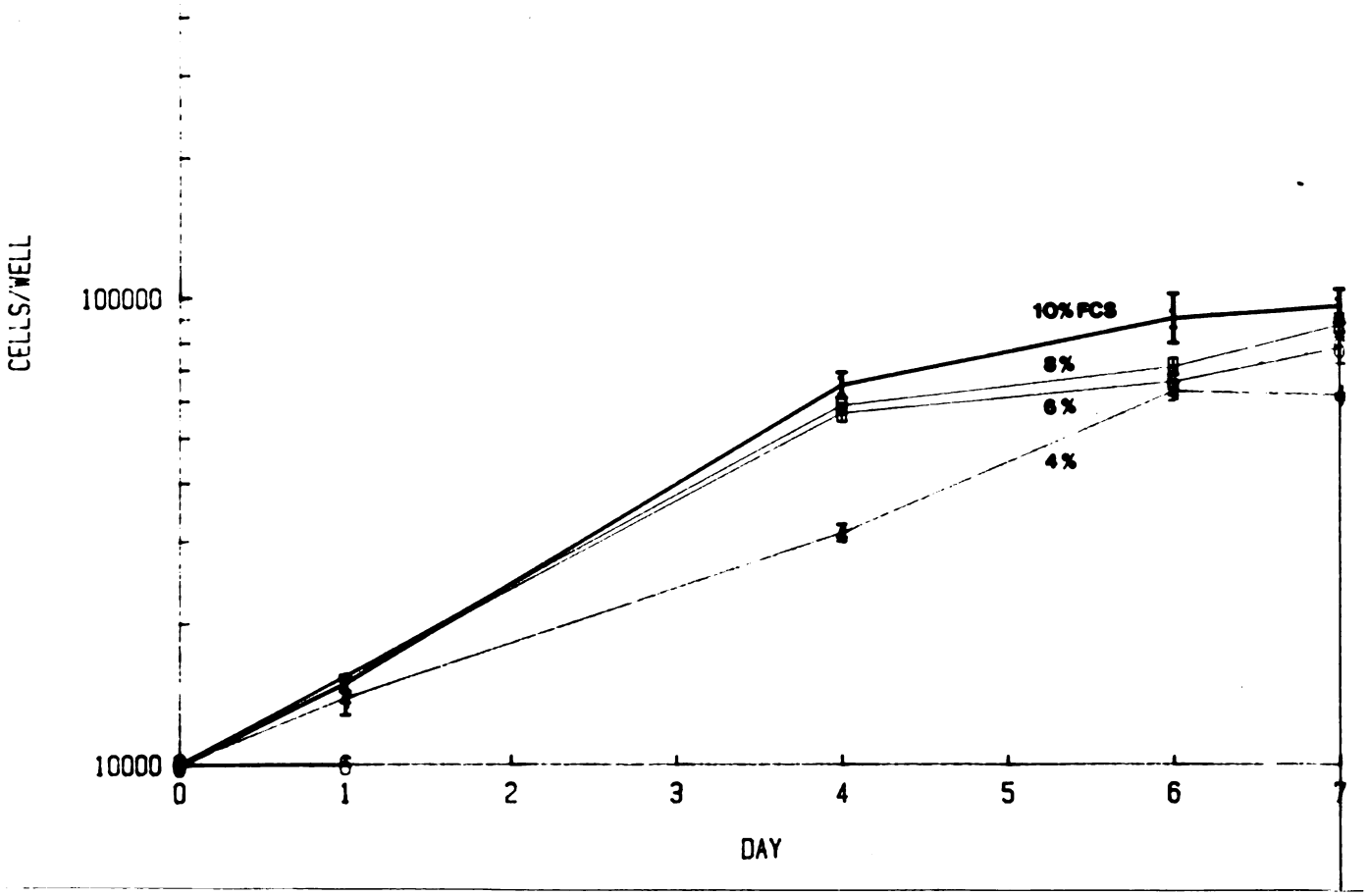
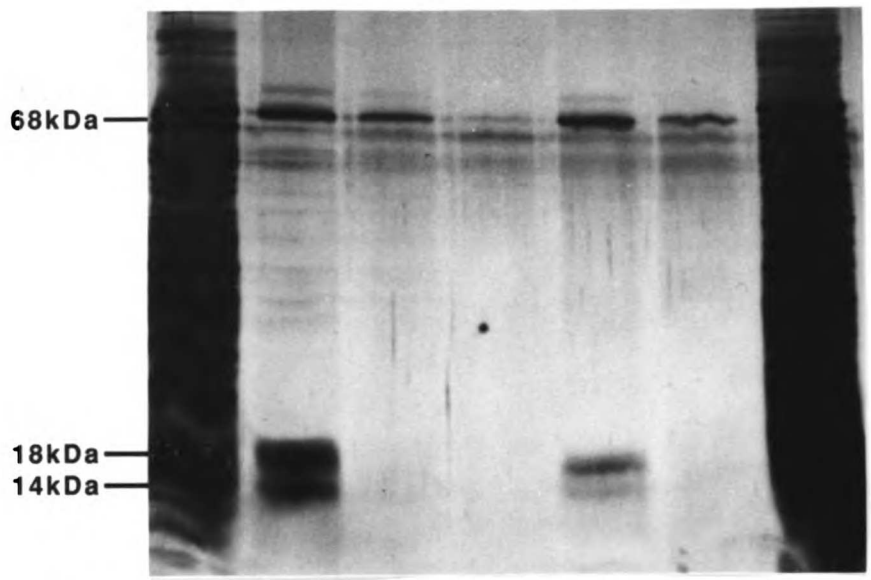


Figure 1b

Figure 2. Electrophoretic profiles of conditioned medium from LPS-stimulated P388D1 macrophages fractionated by heparin-affinity chromatography. Precipitated, heparin-Sepharose fractions were tested for mitogenic activity, and then mitogenic peaks were pooled and electrophoresed on 15% on SDS-PAGE and silver stained. The number of the mitogenic pool (see Chapter 3) and the NaCl concentrations used for elution are shown below each lane. For detailed explanation see Chapter 3, Figure 4g upper panel, 5T and 6T are equal volumes of 5 and 6 after additions of 0.1% Triton X-100, a treatment which prevented loss of bFGF during handling.



Pool#-	2	3	5	6	5T	6T	CM
NaCl M-	0.2	0.4-0.7	1.7-2.1	2.2-2.4	1.7-2.1	2.2-2.4	0.15

Electrophoretic profiles of heparin-affinity fractionated conditioned medium from LPS stimulated P388D1 macrophages.

For detailed explanation see chapter 3 figure 4G and table 2. 5T and 6T are equal volumes of 5 and 6 after solubilization of concentrated medium with 0.1% Triton. Precipitated, concentrated medium was electrophoresed on 15% SDS-PAGE and silver stained.

Figure 2

culture contained greater thatn 98% viable cells, suggesting that cell death is unlikely to be a major source of material in our CM. One possibility is that P388D1 cells may die in culture and be phagocytized (therefore giving a false high viability count). However, efforts to quantitatively extract cellular bFGF have failed (our results and A. Baird, personal communication) and no numerical analysis of this hypothesis is possible. This failure was due to the large residual amount of bFGf that was left in ammonium sulfate pellets after a series of increasing salt concentrations. It was not possible at the time to measure bFGF amount in these pellets.

Direct evidence that bFGF is present in media conditioned by LPS-stimulated P388D1 cells was obtained by a radioreceptor assay (kindly performed by Dr. Gera Neufeld). Pools 5 and 6, but not flow-through or pool 3, appeared to contain small amounts of FGF as they inhibited binding of radiolabeled FGF to its receptor on cells (Neufeld, 1986). We confirmed this conclusion by developing a dot immunoassay (see Chapter 3) with 2 monoclonal antibodies against bovine bFGF peptides (provided by Denis Gospodarowicz). As little as 1 pg of bFGF was detected by this assay, however 20 ng of acidic FGF (aFGF) did not give a positive signal and lysozyme, albumin or DNA also gave no signal (100 ng of each was spotted onto nitrocellulose). Using this dot assay we found that fractions 5 and 6 together from 100 ml of starting CM had a total of approximately 16 ng bFGF (see Table 2 in Chapter 3). Using standard techniques for Western blotting (Towbin et al., 1979, Chapter 3) we demonstrated a major bFGF band migrating at 16 kDa (and minor bands at 14 kDa and 18 kDa) in cell lysates and CM of P388D1 and elicited peritoneal macrophages stimulated with LPS for 24 hr (Fig. 3). Note that differences in molecular weight of putative bFGF in Fig. 2, lane 5T, 6T, and Fig. 3, with bands at 16 kDa and 14 kDa, and Fig. 4b, with a strong band at 18 kDa, are due to differential handling and resultant FGF breakdown in CM. The metabolically labelled material giving a strong 18 kDa band in Fig. 4b was applied directly to the heparin-Sepharose

Figure 3. Demonstration of bFGF in lysates and secretions of LPS-stimulated peritoneal and P388D1 macrophages. Western blot of precipitated proteins present in the CM and cell lysates of peritoneal and P388D1 macrophages. Lane at far left is bovine basic FGF control (D. Gospodarowicz). Lane 1=P388D1 CM cell lysates 2=P388D1, 3=peritoneal macrophage CM, 4=peritoneal macrophage cell lysates. All cells were treated for 48 hours with 10 μg/ml of LPS. A mixture of two anti-bovine basic FGF peptide monoclonals were used at a concentration of 1/1000. Acidic FGF antigen was negative in this transfer (not shown). Control FGF dot blots reacted with second antibody alone were also negative.

Stimulated peritoneal and P388D1 macrophages synthesize basic FGF.

Figure 3

column without dialysis and lyophilization, whereas those preps in Fig. 2 and Fig. 3. (giving putative 14 kDa and 16 kDa breakdown products from the 18 kDa band) underwent dialysis and lyophilization before reconstitution and application to the heparin-Sepharose column.

Because the bFGF monoclonal antibodies were not able to immunoprecipitate bFGF we tried to determine whether macrophges biosynthesized bFGF by biosynthethic labelling of the cells, collection of the radiolabelled proteins in their CM followed by fractionation of the CM by heparin-Sepharose affinity chromatography. We then attempted to correlate the appearance of 14-18 kDa bands in the heparin-binding column fractions with mitogenic activity. Adherent P388D1 cells were stimulated with LPS in 10 ml of serum-free and cysteine-free medium containing 100 μCi of ^{35}S-cysteine (specific activity 691 Ci/mmol) for 24 hr. The CM was collected, and then applied to a heparin-Sepharose column. The loading buffer was run through the column until less than 100 cpm/10 μl was detected in eluant, and then a 20 ml gradient of phosphate-buffered 0.15-3.0 M NaCl was applied. 1 ml fractions were collected, dialyzed against 3 changes of 1 liter PBS at 4 C, and then the frractions were assayed for (1) cpm=(protein), (2) mitogenic activity, and (3) intensity of 14-18 kDa migrating bands detected be autoradiography after 15% SDS-PAGE. Mitogenic activity correlated with increased strength of the radiolabelled 14-18 kDa bands (Fig. 4a,4b). These bands correlated with significant mitogenic peaks in three separate experiments. The 14-18 kDa bands correspond with bFGF in size and heparin affinity (determined by control elutions) and could account for 50-70% of labelled protein in these fractions (determined by scanning densitometry). Taken together with the data indicating immunoreactive FGF in similar heparin-affinity fractions, we infer that macrophages synthesize and release mitogenic bFGF into CM.

As another approach to demonstrating that P388D1 MØ cells do make bFGF, we determined whether mRNA for bFGF was present in macrophages. Northern blots of RNA

Figure 4. (a) Heparin-Sepharose elution profile of secreted proteins collected from radiolabeled, LPS-stimulated P388D1. 1 ml column fractions were collected. The fraction numbers are indicated on the abscissa, curve A is the concentration of NaCl used for elution (right ordinate), E=CPM of ^{3}H-thymidine uptake induced by dialyzed column fractions in a Balb/c3T3 mitogenesis assay, D=protein content in arbitrary units as indicated by CPM of ^{35}S-cysteine incorporated into protein as analyzed in column fractions dialyzed in 2K cut-off membranes. Lines B and C are the positive (10% serum) and negative controls (10% medium) in the mitogenesis assay. (b) Quinine sulfate precipitated proteins from individual fractions as indicated in Fig. 4a were fractionated by 15% SDS-PAGE under reducing conditions, and autoradiographed. Fraction number, NaCl concentration, and mitogenic activity are indicated below each lane. Arrowheads indicate the enriched 14-18 kDa bands found in heparin-binding proteins of the mitogenic fractions 17 and 19.

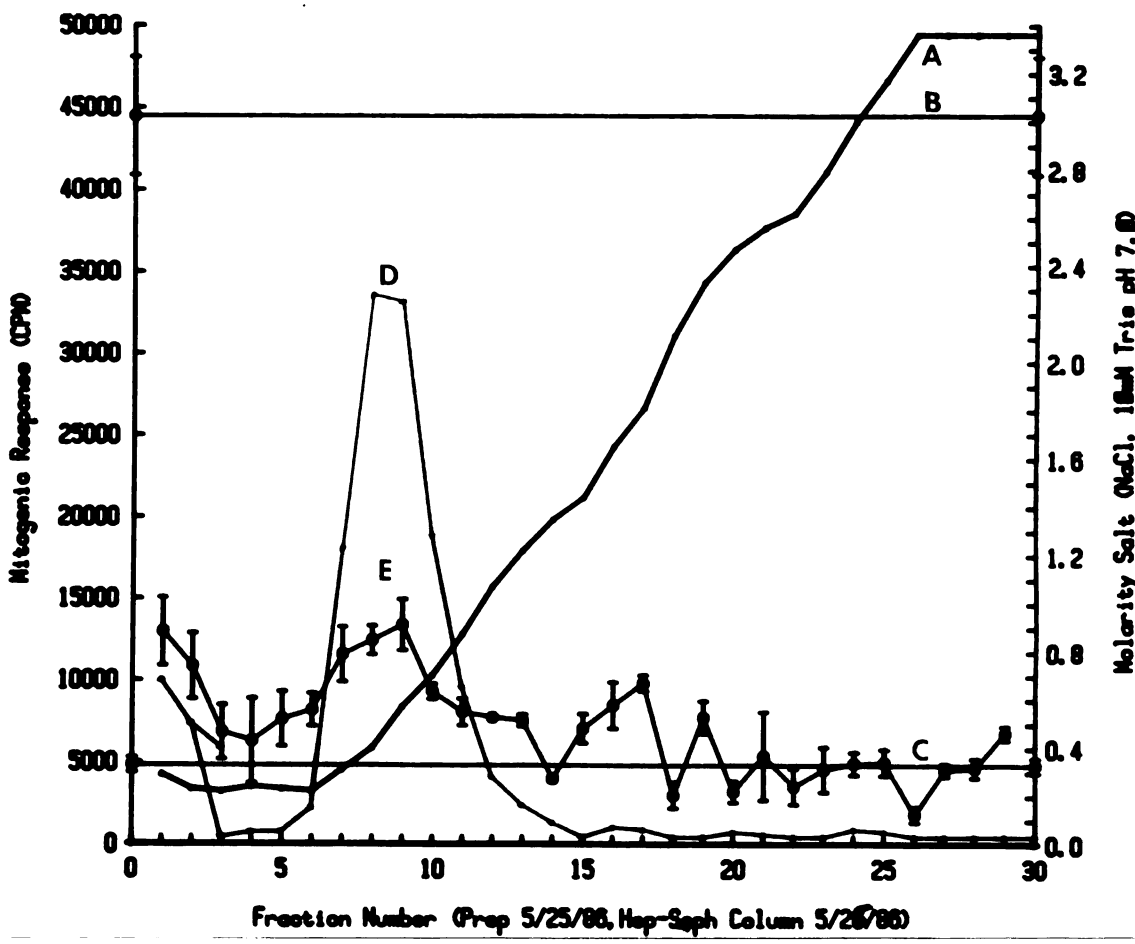
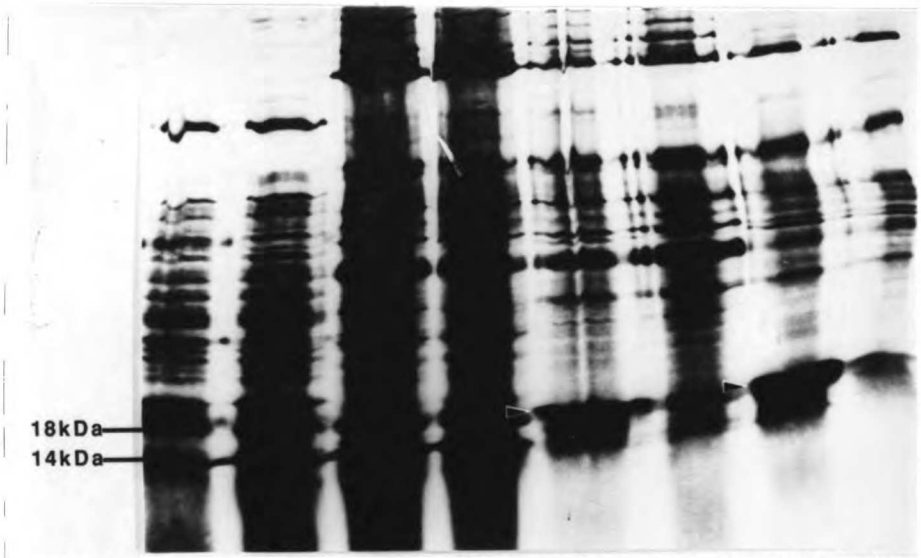

50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Mitogenic Response (CPM)
A
B
C
D
E
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Molarity Salt (NaCl, 10mM Tris pH 7.0)
0
5
10
15
20
25
30
Fraction Number (Prep 5/25/86, Hep-Seph Column 5/26/86)

Figure 4a



P388D1 macrophages synthesize and secrete a mitogenic activity with heparin affinity corresponding to basic FGF.

Figure 4b

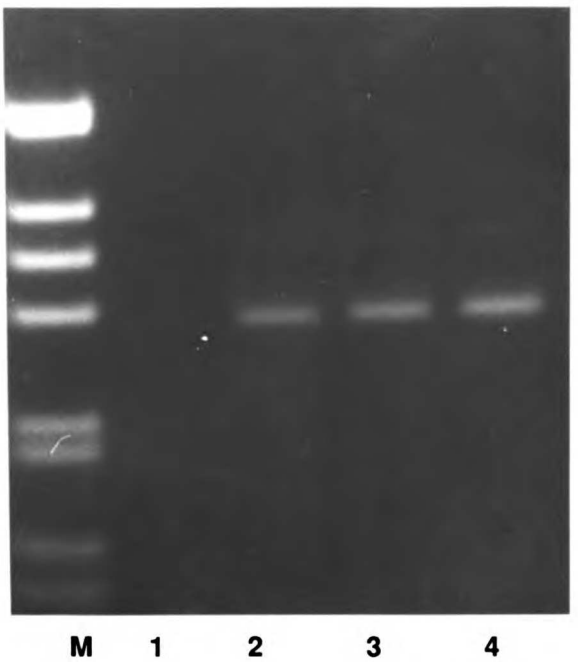
from LPS-stimulated and unstimulated P388D1 macrophages and elicited peritoneal macrophages were hybridized with bFGF cDNA labelled by DNA synthesis with random priming (10^8 cpm/μg) and 3.6 and 7.0 kb bFGF transcripts were found in both stimulated and unstimulated P388D1 macrophages in similar amounts (under stringent wash conditions, data not shown). In addition, we have recently demonstrated bFGF mRNA transcripts in anemia-induced erythroid-clustering macrophages, as well as in LPS-stimulated P388D1 macrophages, and mouse brain (Fig. 5) by the reverse transcriptase-polymerase chain reaction technique (see Chapter 2). The erythroid-clustering macrophages were purified by a technique developed by Paul Crocker (1986). Very little is known regarding the regulation of bFGF accumulation. Indeed, the hypoxia correlated with anemia may be a modulator of FGF transcription in differentiated cells since we found FGF mRNA in macrophages from anemic, but not normal mice. However, the RT-PCR was not quantitative and the amount of bFGF transcript accumulation must be determined. Since hypoxia is a wound condition, and bFGF is a wound healing growth factor, a quantitative approach is of interest. The study therefore requires development of a quantitative RT-PCR.

We conclude that macrophages synthesize and liberate bFGF polypeptide and transcribe bFGF mRNA. The amount of bFGF liberated by 10^6 macrophages/ml/day is mitogenic for Balb/c 3T3 fibroblasts and we speculate that liberation of bFGF by wound macrophages contributes to wound healing processes in vivo.

Epidermal Growth Factor

The largest peak of fibroblast mitogenic activity from medium conditioned by LPS-stimulated P388D1 macrophages was in the flow through of the heparin affinity column. Because elicited LPS-stimulated peritoneal macrophages do not secrete a mitogenic activity of strong heparin affinity, and the mitogenic activity with the weak heparin binding contained PDGF (Table II, Chapter 3), we pursued the identity of the mitogen(s) that does not bind to

Figure 5. Macrophages contain mRNA transcripts for basic FGF. RT-PCR products were electrophoresed in 4% agarose and stained with ethidium bromide. Lane M=1 kb ladder, 1=oligonucleotide primers only, 2=LPS-stimulated P388D1 macrophages, 3=erythroid precursor-agglutinating macrophages from the spleens of the anemic mice (brightest 10% of SER-4- and F4/80-positive macrophages isolated by fluorescent activated cell sorting (see Chapter 3). 4=mouse brain used as a positive control.

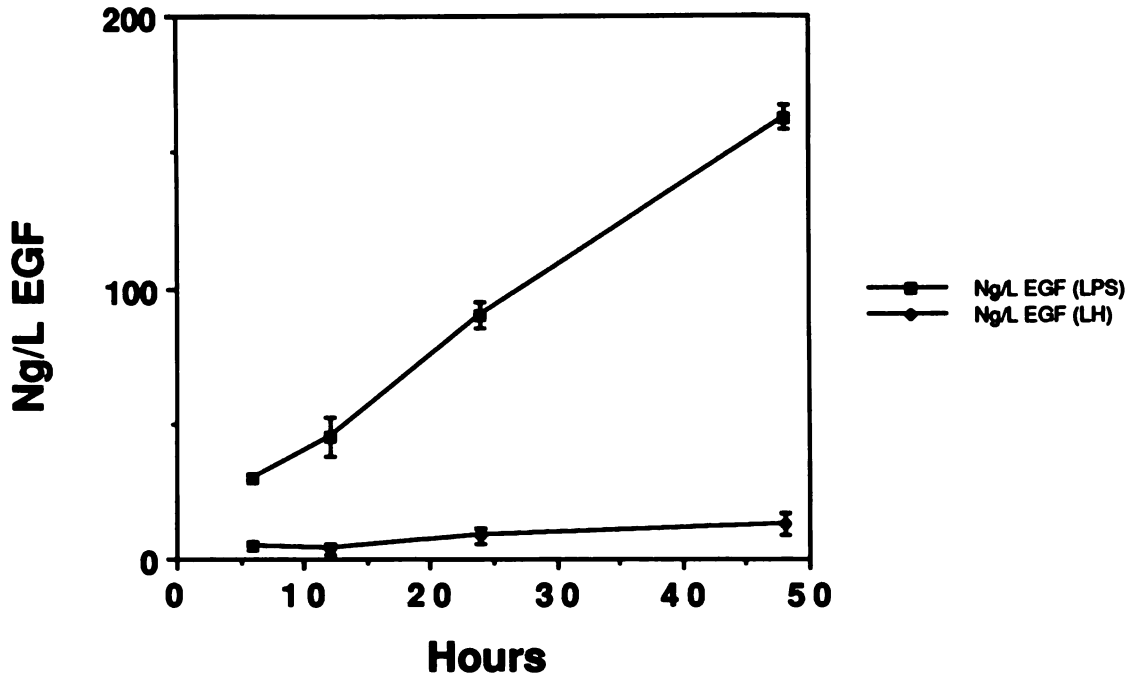


Macrophages transcribe basic FGF.

Figure 5

heparin. First, a competitive EGF/TGF-α radioreceptor assay (Biomedical Technology, Inc.) was used to determine how much EGF and TGF-α was present. 100 ml of CM from P388D1 stimulated with LPS for 48 hr were found to contain 25-35 ng of EGF/TGF-α. Since net mitogenic activity is a property of interactions of growth factors and inhibitors we have not yet tried to determine whether other growth factors contribute to the mitogenic acitivity of the flow-through. Equivalent amounts of EGF added to the mitogenesis assay induced similar mitogenic responses, however. The EGF receptor has equal Kd for TGF-α and EGF ligands (Carpenter, 1986), and therefore can not distinguish between EGF and TGF-α. To distinguish EGF and TGF-α, a direct non-competitive ELISA was developed using Collaborative Research polyclonal anti-mouse EGF antiserum, which does not react with TGF-α under conditions described for the TGF-α ELISA in Chapter 3. Stimulus- and time-dependent accumulation of EGF antigen was observed in unfractionated CM of thioglycollate-elicited peritoneal macrophages stimulated with LPS, but not in CM of unstimulated macrophages (Fig. 6). Western blot analysis of the CM from LPS stimulated peritoneal macrophages revealed the presence of authentic 6 kDa EGF. The 6 kDa immunoreactive EGF was found at much higher concentrations in CM of LPS stimulated P388D1 and peritoneal macrophages, than in CM of unstimulated macrophages. The anti-EGF antiserum did not detect TGF-α dotted onto the same nitrocellulose sheet that contained blotted SDS-PAGE fractionated macrophage CM (Fig. 7). The specificity of the antibody is additionally important since EGF and TGF-α are similar size (6-7 kDa) and might not be resolved in the 15% SDS-PAGE. A fourth line of evidence suggesting production of EGF by macrophages was the positive immunofluorescent signals seen in stimulated, but not unstimulated, macrophages in serum-free culture (Fig. 8). These experiments were performed as described for TGF-α in Chapter 3, with male mouse submaxillary gland cells used as a positive control. The cellular EGF immunofluorescence was blocked by 1,000 fold

Figure 6. LPS-stimulated macrophages liberate EGF in a time- and stimulus-dependent manner. Elicited peritoneal macrophages were cultured in the presence (LPS) or absence (LH) of 10 μg/ml LPS in serum-free medium, and conditioned medium was collected after 6, 12, 24, and 48 hours (abscissa). LH= a hydrolysate of lactalbumin was a non-stimulatory medium. Triplicate samples were assayed by a direct non-competitive ELISA described in the text. EGF concentration in macrophage CM is indicated on ordinate. Error bars indicate standard error of the mean.



Stimulated macrophages liberate EGF in a time- and stimulus-dependent manner.

Figure 6

Figure 7. LPS-stimulated macrophages liberate EGF into conditioned medium. Western blot of macrophage secreted proteins. Lane 1=EGF (Collaborative Research), 2=1/100 dilution of submaxillary gland lysates as a positive control, 3,4= CM from LPS-stimulated elicited peritoneal macrophages 5,6= CM from unstimulated elicited peritoneal macrophage 7,8= CM from LPS-stimulated P388D1 9,10= CM from unstimulated P388D1 conditioned medium. The Western transfer of precipitated media electrophoresed by 15% SDS-PAGE. Anti-EGF diluted 1/200 (Collaborative Research) was used as a first antibody. Dot blots using irrelevant antigens or second antibody only were negative. Total protein loaded in all lanes did not vary by > 10%.

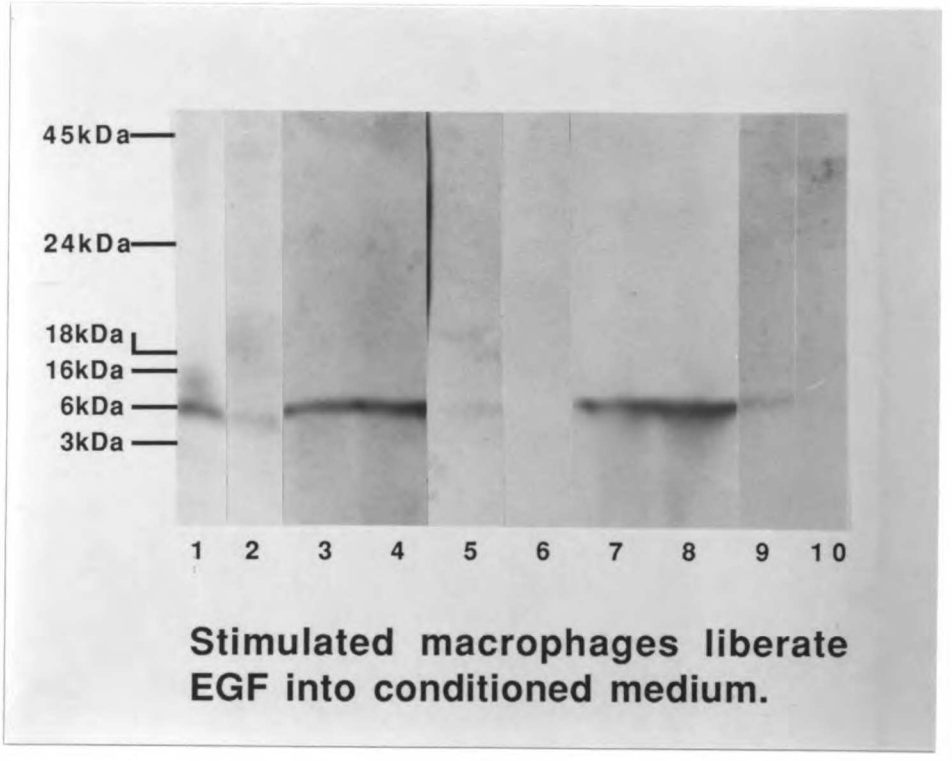

45kDa
24kDa
18kDa
16kDa
6kDa
3kDa
1 2 3 4 5 6 7 8 9 10
Stimulated macrophages liberate EGF into conditioned medium.

Figure 7

Figure 8. LPS-stimulated bone marrow-derived macrophages express EGF antigen. All immunofluorescence exposures at 250 x magnification under similar lighting conditions during microscopy and photography. (See Chapter 3 for a full description of conditions). a=LPS-stimulated macrophages stained with anti-EGF diluted 1/250 (Collaborative Research), b=LPS-stimulated macrophages stained with normal rabbit serum diluted 1/250, c=submaxillary gland cells stained with 1/250th anti-EGF diluted 1/250 as in a, d=submaxillary gland cells stained with normal rabbit serum as in b, e=unstimulated macrophages stained with anti-EGF as in a, f=LPS-stimulated macrophages stained with anti-EGF as in a, in the presence of 1,000-fold stoichiometric excess of EGF (Collaborative Research).

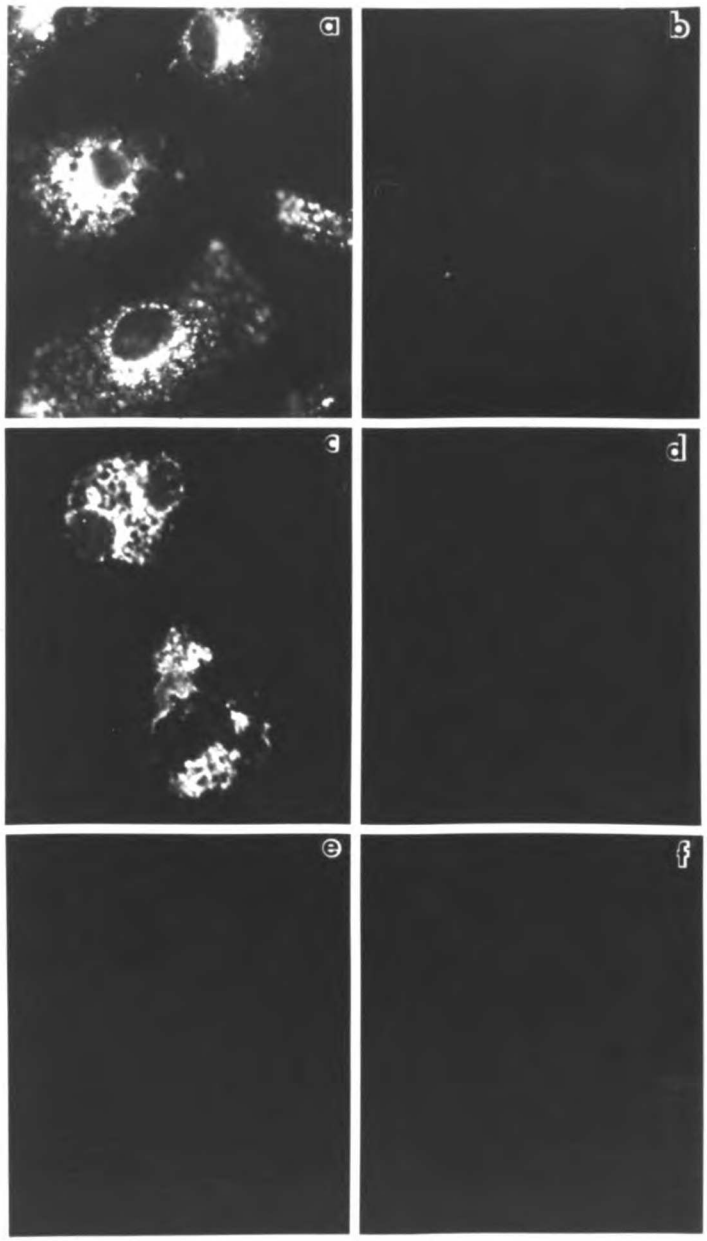


Figure 8

excess of EGF antigen. Positive signals appeared in greater than 90% of stimulated macrophages. Less than 10% of unstimulated macrophages were positive by immunofluorescence and in these cells the signals were weaker, requiring greater than 10-fold exposure times to obtain a signal approaching that of stimulated macrophages. These results suggested that macrophages are induced by LPS to make and secrete EGF. However, we were unable to demonstrate EGF biosynthesis by radiolabelling macrophages with ^{35}S-cysteine, then collecting the CM and immunoprecipitating EGF with EGF antiserum (Collaborative Research) that was capable of immunoprecipitation of ^{125}I-EGF. Therefore, there were several lines of evidence suggesting that macrophages contained and liberated EGF polypeptide, but evidence agianst direct synthesis of EGF polypeptide.

Further evidence against macrophage synthesis of EGF was obtained by Northern blot and RT-PCR (reverse transcription polymerase chain reaction) analysis of mRNA from stimulated or unstimulated P388D1 or thioglycollate elicited peritoneal macrophages. Northern blot analysis of 10 μg of whole cellular macrophage RNA yielded no 4.5 kb EGF mRNA whereas 10 μg of whole cellular RNA from hypoxic kidney or 0.1 μg (Fig. 9a) or 0.2 μg (Fig. 9b) male submaxillary gland whole cellular RNA yielded a detectable signal. Because male mouse submaxillary glands contain 100-1,000 transcripts/cell (Gray et al., 1984), and yield a signal with 1/100th the amount of RNA compared to that assyaed for macrophage, there would be a limit of detection less than 2-10 copies of EGF transcript per macrophage. Since macrophages are small cells (yielding only 2.5 pg whole RNA/cell) it is likely that macrophages would theoretically contain less than 1 EGF transcript per cell by this type of analysis.

The single cell mRNA phenotyping method was also used to assay many macrophage RNA preparations for EGF transcripts. The detection threshold for our EGF oligonucleotides is between 10-100 pg submaxillary gland RNA (i.e. 200-10,000 EGF

Figure 9. Macrophages do not contain EGF mRNA transcripts by Northern blotting analysis. Northern blot hybridized with random primed EGF cDNA (Pharmacia), and washed at 1xSSC, 65°C, 0.1% SDS, 1 hour. Except submaxillary gland positive controls (a. lane 9-0.1 μg and b. lane 4=2.0 μg) all lanes have 10 μg total cellular RNA. a. 1,3=LPS stimulated P388D1 and elicited peritoneal macrophages, respectively, 2,4=unstimulated P388D1 and elicited peritoneal macrophages, respectively, 5,6=hypoxic and normoxic rabbit macrophages, 7=hypoxic kidney, 8=brain, 9=submaxillary gland. b. 1=brain, 2=hypoxic kidney, 3=liver, 4=submaxillary gland, 5,6=LPS-stimulated and unstimulated P388D1 macrophages, 7,8=LPS-stimulated and unstimulated elicited peritoneal macrophages. Arrowheads indicate position of 4.5 kb EGF transcript.

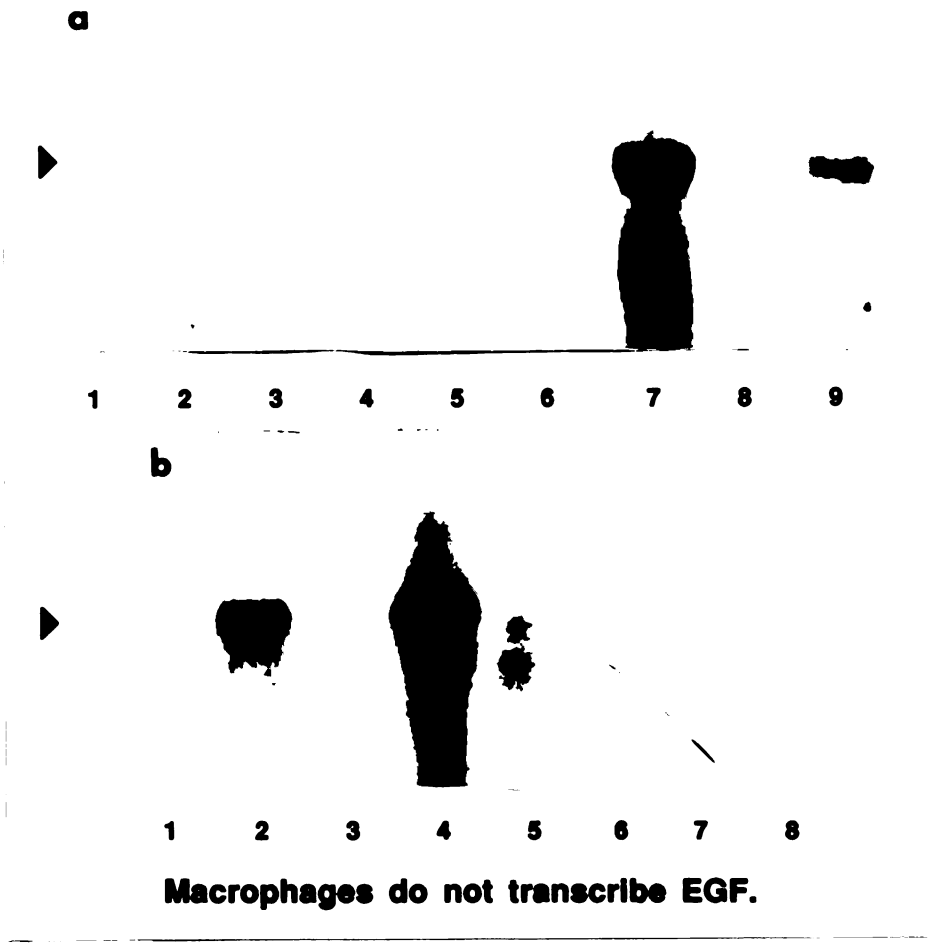
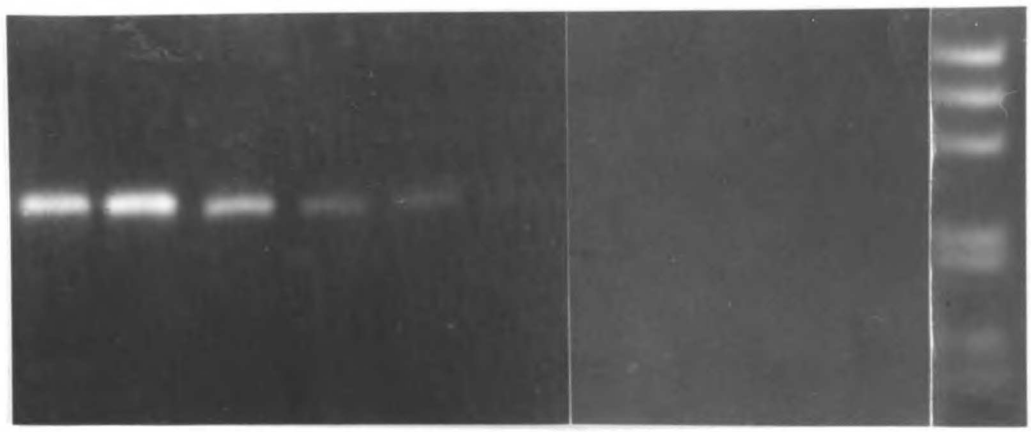


Figure 9

transcripts), but 250,000 macrophages failed to produce and EGF signal in the RT-PCR (Fig. 10). If EGF transcripts are present, there is less than 1 transcript/macrophage. An experiment mixing macrophage RNA plus submaxillary gland RNA was not done, and thus possible EGF transcript blocking effects by macrophage RNA cannot be definitively ruled out. It should be noted, however, that many other growth factor mRNA transcripts present in low copy number were detected in macrophage RNA preparations negative for EGF signals.

There are several interpretations of these conflicting data. One possibility is that stimulated macrophages liberate EGF from culture plastic which has previously been exposed to fetal calf serum. We did not see EGF polypeptide liberated by LPS-stimulated elicited peritoneal macrophages which were cultured in the complete absence of fetal calf serum. EGF was liberated by the macrophages only when the initial phase of culture was done in the presence of serum. It is interesting to note that we also find TGF-α, but not EGF transcripts in another bone marrow cell, megakaryocytes (see Appendix B), and conclude that EGF in serum must also be present in plasma, since megakaryocyte-derived platelets are therefore unlikey to be the source of EGF in serum. We have found no macrophage EGF receptor (data not shown), but EGF may be taken up during monocyte notogeny through a bulk phase endocytosis mechanism similar to the case for albumin uptake by megakaryocytes. Alternately, serum EGF bound to a plasma protein may be taken up by a receptor-mediated process. Such EGF, present within the endocytic pathway and trans-Golgi network, may be the source of intracellular EGF. Alternatively, another molecule may account for the immunofluorescence signal in stimulated macrophages cultured on plastic never exposed to fetal calf serum. We intend to test the EGF liberation hypothesis be adding ^{125}I-EGF, or ^{125}I-albumin as a specificity control, to serum, absorb this to culture plastic wash the plastic and test liberation of radiolabelled protein into CM of LPS stimulated or unstimulated macrophages. An alternative experiment is suggested by the observation that PDGF and

Figure 10. Macrophages do not contain EGF mRNA transcripts as determined by RT-PCR. Lanes 1-6 are a dilution series of mouse male submaxillary gland RNA. Respectively there are 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 picograms of RNA used in the RT-PCR. 7,8=LPS-stimulated and unstimulated P388D1 macrophages, 9,10=LPS-stimulated nad unstimulated peritoneal macrophages. Each of the RNA preps in 7-10 were positive for TGF-β; M= 1kb ladder.



Macrophages do not transcribe EGF mRNA.

Figure 10

TGF-β are non-covalently bound to α_2-macroglobulin (α_2M) (Ross, personal communication, O'Connor-McCourt, 1987), which is found in plasma and also is a macrophage secretion product (Hovi et al., 1977). α_2M-protease complexes are internalized by macrophages by receptor-mediated endocytosis. It is not possible to trypsinize macrophages off plastic, but P388D1 are viable when cultured in suspension. Therefore, to test for uptake of ^{125}I-EGF by the α_2M transport mechanism, the radiolabelled EGF will be mixed with α_2-M in both high uptake and low uptake forms and added to LPS-stimulated or unstimulated P388D1 in suspension culture. Equal numbers of stimulated or unstimulated P388D1 will be assayed for radioactive content to determine EGF uptake. These experiments should determine whether LPS-stimulated and or unstimulated macrophages have the ability to take up and liberate exogenous EGF.

An effort will be made to determine whether the immunoreactive EGF liberated by macrophages is mitogenic. CM from LPS-stimulated and unstimulated macrophages cultured under conditions where EGF is liberated will be tested for mitogenic activity in the bioassay previously described. CM with mitogenic activity will be passed over protein A-Sepharose columns coupled with anti-EGF IgG or non-immune IgG. The non-binding fractions will be tested for mitogenic activity. Decreased mitogenic activity in fractions flowing through the anti-EGF affinity column (checked over a dilution series and compared with a dilution series prepared before chromatography) compared to fractions from the nonimmune IgG affinity column would indicate a possible contribution of EGF to the mitogenic activity in macrophage CM. These data would be substantiated if EGF mitogenic activity could be eluted from the anti-EGF affinity column by low pH, high pH, or urea. A second approach is to separate immunoreactive EGF on non-denaturing PAGE and then electroelute immunoreactive EGF and directly test its mitogenic activity.

Nerve Growth Factor-β

Crush injury of the peripheral nerves, but not central nervous system leads to rapid demyelination followed by nerve regeneration. Macrophages may be important in removal of myelin in the degenerating peripheral axon (Beuche and Friede 1984). It was shown recently that macrophages infiltrate crushed peripheral, but not central nervous system, nerve sheath at a time (4 days after crush) that precedes and concurs with the period of maximal Schwann cell growth (Perry). Macrophages have been implicated in nerve regeneration by production of nutritive molecules such as apolipoprotein E (ApoE), which binds to lipid, and trophic molecules such as IL-1. ApoE may be important in the later stages of nerve regeneration: this macrophage secretion product approaches 5% of total secreted protein of regenerating nerve at 3 weeks post crush (Muller et al., 1986), and is taken up at nerve growth cones after NGF stimulation (Ignatius et al., 1986). NGF transcription in degenerating crushed sciatic nerves occurs in two peaks: a transient immediate peak, and a second chronic peak seen in vivo 4 days after crush, (Perry *et al.*, Beuche and Friede, 1986). In vitro, crushed sciatic nerve displays the second NGF mRNA peak only after the addition of activated macrophages (Heumann, 1987). IL-1 induces NGF mRNA in nerve segments in vitro, and, thus, it may be the major effector of regenerating nerve NGF mRNA induction, although PDGF and TNF-α have smaller inductive effects. FGF has no effect on NGF mRNA induction (Lindholm et al., 1987).

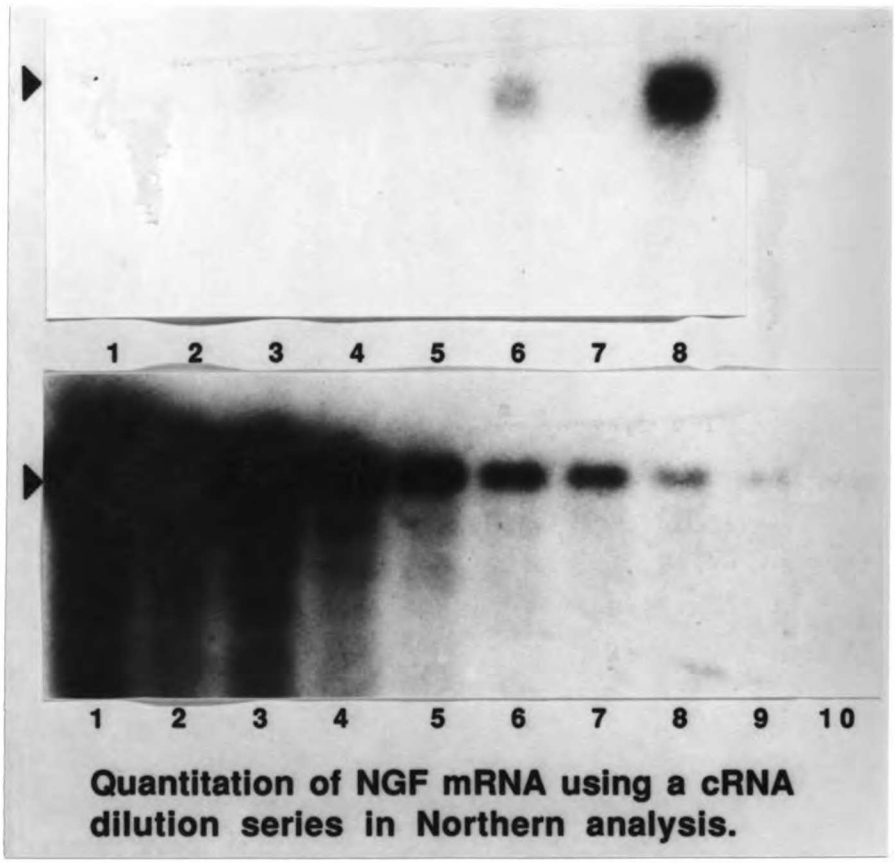
We wanted to know whether the requirement for NGF production in crushed nerve reflected synthesis of NGF my MØ or if some ohter mechanism was in operation. Therefore, we looked for macrophage production of NGF. RNA from stimulated and unstimulated macrophages, was purified by 3 cycles of oligo-dT affinity chromatography, (as per manufacturere instructions, Miles) and then fractionated by 1.2% agarose gel electrophoresis under denaturating conditions, transferred to nylon membranes and hybridized with single-stranded NGF cDNA generated from an M13 vector. The macrophage RNA samples were

corrected for macrophage RNA yields. The amount of hybidizing mRNA was compared to a dilution series of SP6 NGF sense strand cRNA run on the same gel (Fig. 11). Data from several quantitative Northern blots suggested that production of NGF mRNA by elicited peritoneal macrophages was very low (< 1 copy cell). In addition, we were unable to find NGF mRNA in macrophage cell lines. Fibroblasts can produce up to 200 copies NGF mRNA cell (Clegg, unpublished results). Thus there was a good possibility that NGF was produced by the 1-10 contaminating fibroblasts per 1000 cells in the macrophage preparations.

We then compared NGF mRNA and NGF protein in macrophages cultures. Cultures of LPS-stimulated peritoneal macrophages contaminated with 1 fibroblast/100 macrophages produced NGF transcripts and protein in a time-dependent manner (Fig. 12). However, the NGF transcript and polypeptide production normalized to macrophage number was exceedingly low (< 1 molecule of NGF mRNA/macrophage and 2 NGF polypeptides/macrophage/day).

To ascertain whether macrophages contained very low levels of NGF mRNA we analyzed macrophage RNA preparations by RT-PCR. To give a rough idea of the minimum threshold of the assay, NGF copy RNA was diluted in a log base 10 series, added to 1 μg E. coli rRNA, and reverse transcribed by priming with the specific NGF 3′ oligonucleotide priming. As few as 10-100 copies of input RNA were detectable following polymerase chain reaction. When RNA from 50,000 LPS-stimulated or unstimulated P388D1 or peritoneal macrophages produced no NGF signal (Fig. 13). The peritoneal macrophage cultures were treated with trypsin (an enzyme which causes fibroblasts to detach but macrophages to flatten out) and washed free of fibroblasts (by visual inspection). The data suggested that macrophages do not contain NGF mRNA. NGF mRNA was never detected by RT-PCR in FACS purified LPS-stimulated peritoneal macrophages also (data not shown). This suggests that another cell type contaminating peritoneal macrophage cultures, such as fibroblasts,

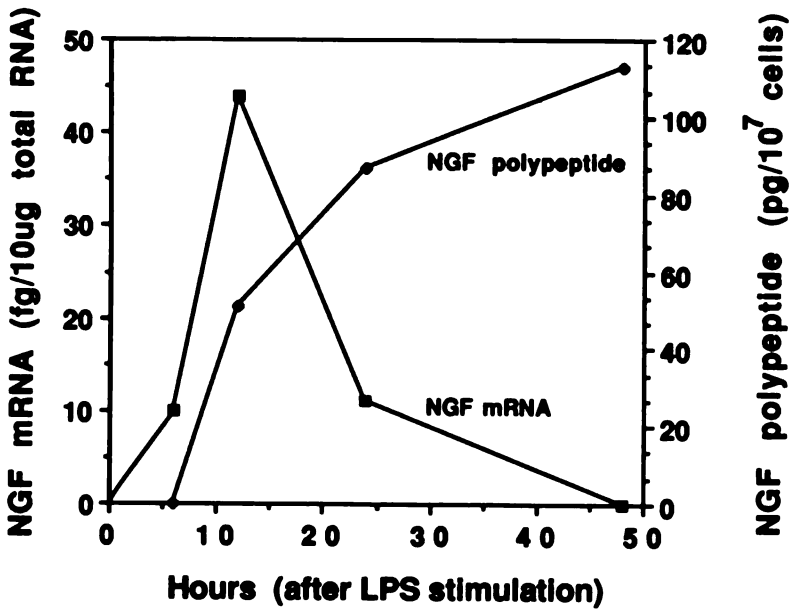
Figure 11. Quantitation of NGF-β mRNA using a cRNA dilution series in Northern analysis. All lanes have 2.5 μg of 3 x oligo-dT purified poly A+ RNA (checked after transfer by oligo dT hybridization). Top panel; Lane 1,2=LPS-stimulated P388D1, 3,5=LPS-stimulated elicited peritoneal macrophages, 4=unstimulated P388D1 macrophages, 6=unstimulated elicited peritoneal macrophages, 7=LPS stimulated endothelial cells, 8=submaxillary gland. Lower panel: A dilution series of SP6 generated NGF cRNA. Lane 1=59.2 pg, 2=29.6 pg, 3=14.8 pg, 4=7.4 pg, 5=3.7 pg, 6=1.85 pg, 7=0.975 pg, 8=0.463 pg, 9=0.23 pg, 10=0.116 pg (not shown 11=58 pg). Arrowheads indicate position of 1.3 kb NGF-β message.



Quantitation of NGF mRNA using a cRNA dilution series in Northern analysis.

Figure 11

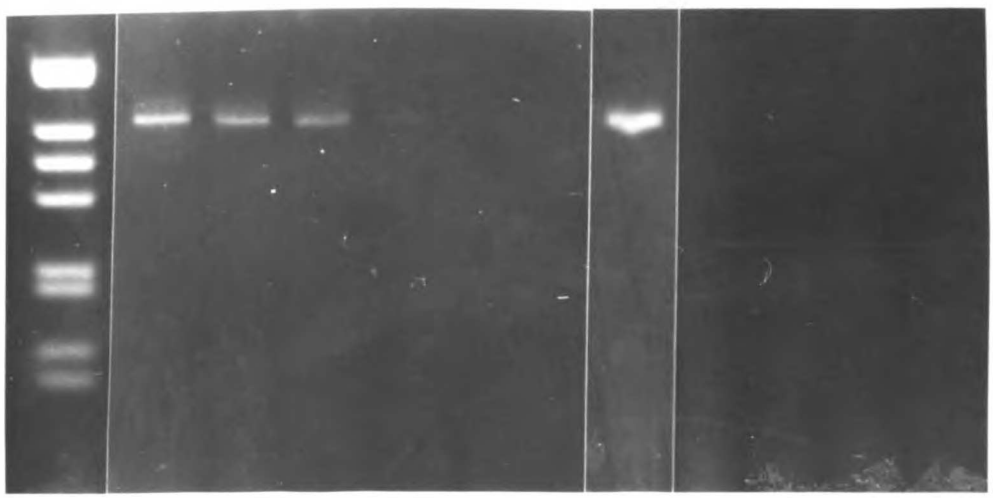
Figure 12. LPS-stimulated macrophages induce NGF mRNA and polypeptide production in contaminating peritoneal fibroblasts. Elicited peritoneal macrophage were cultured in the presence of 10 μg/ml LPS. Cells and CM were removed at 6, 12, 24, and 48 hours (see abscissa). NGF transcript was quantified by method outlined in text and in Figure 11. NGF polypeptide was assayed by a two site ELISA (Dr. Dennis Clegg).



Stimulated macrophages induce NGF mRNA and polypeptide production by contaminating peritoneal fibroblasts.

Figure 12

Figure 13. Macrophages do not transcribe NGF mRNA. M=1 kb ladder, lanes 1-6 are a dilution series of SP6 generated NGF-β cRNA; 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 copies, respectively. 7=Balb/c 3T3 fibroblast NGF positive control, 8=unstimulated peritoneal macrophage, 9=LPS-stimulated peritoneal macrophages, 10=unstimulated P388D1 macrophages, 11=LPS-stimulated P388D1 macrophages. In 8-11, macrophages were cultured for 10 hours, with or without stimuli, before RNA was processed.



Macrophages do not transcribe NGF mRNA.

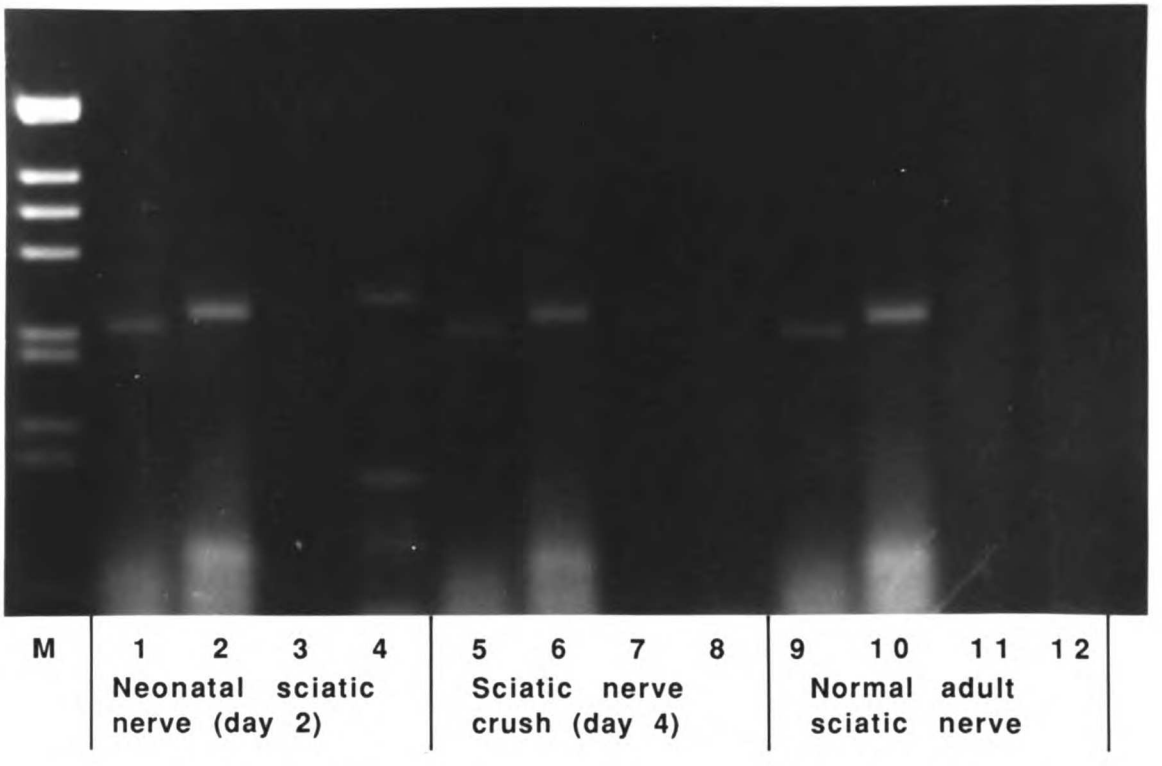
Figure 13

makes NGF, either directly in response ot LPS, or indirectly in response to a mediator made by macrophages in response to LPS. In this respect, it is intteresting that acetylated LDL, which enters by a specific receptor, and produces lipid loading in macrophages, but not fibroblasts (Brown and Goldstein, 1985), stimulated NGF transcription in fibroblast-contaminated peritoneal macrophage cultures. This suggested that acetylated LDL stimulated macrophages were releasing a factor which induced transcription of NGF by fibroblasts.

We then tried to elucidate the role of macrophages in regulating NGF mRNA production in the contaminated macrophage preparations. We observed that acetylated LDL also stimulated macrophages to transcribe IL-1α, TGF-α, M-CSF, and G-CSF (data not shown). Thus, macrophages might be capable of effecting NGF transcription and translation in contaminating fibroblasts by one of these factors. Indeed it has been reported that IL-1 induces NGF transcript accumulation in crushed nerve in vitro (Lindholm *et al.*, 1987). One goal is to determine which growth factors induced by lipid uptake by macrophages induce NGF synthesis by fibroblasts.

Nerve growth occurs normally during growth and development, as well as during repair. If there are mechanistic similarities in these two functionally similar situations, then the phenotype of growth factor transcripts in regenerating, and in normally growing neonatal sciatic nerve may be similar. Sciatic nerve was surgically exposed, crushed, resituated, and the 2 mm of the distal nerve stump was removed 4 days later (a technique kindly taught to me by Dr. Mike Ignatius). Segments of 2 mm from neonatal, crushed, and contralateral sham-operated sciatic nerves was single cell mRNA phenotyped by the method described in chapter 2. Actin and PDGF-A transcripts were found in normal adult and neonatal nerve, as well as in crushed nerve, whereas TGF-α was seen only under conditions of nerve growth and repair, but not in adult nerve (Fig. 14). Interestingly, EGF transcripts were also found in neonatal nerve, but not in normal or crushed adult nerve. To date we have not seen IL1-α transcripts

Figure 14. Growth factor mRNA transcripts are expressed in neonatal and regenerating sciatic nerve. Neonatal nerve at day 2 post-partum, crushed sciatic nerve at day 4 after injury, and normal sham-operated sciatic nerve were collected in 2 mm segments and processed for RT-PCR as described in text. M=1 kb ladder, lanes 1, 5, 9=PDGF A, 2, 6, 10=β-actin, 3, 7, 11=TGF-α, and 4, 8, 12=EGF.



TGF-alpha transcript is expressed in neonatal and regenerating sciatic nerve.

Figure 14

in nerve. These data suggest that nerve repair and neonatal growth may utilize different growth factors.

Our goals for the future, include: (a) scanning crushed nerve for a complete growth factor phenotype; (b) adding the polypeptides indicated by (a) to fibroblasts and assaying for NGF mRNA induction; and (c) blocking fibroblast NGF mRNA induction by addition of blocking growth factor antiserum to fibroblast cultures. By these means we hope to correlate growth factor and nutritive factor phenotypes in vivo with function of macrophage-derived factors in NGF mRNA induction. Another goal is to isolate macrophages from crushed nerve segments and determine their growth factor phenotype directly. In this way, we hope to better understand the role macrophages play in nerve regeneration.

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Appendix A

Platelet-derived Growth Factor, Transforming Growth Factor-α and Transforming Growth Factor-β Genes are Expressed During Development in Preimplantation Mouse Embryos.

Summary

Control of growth and differentiation during mammalian embryogenesis may be regulated by growth factors from embryonic or maternal sources. Using a novel method for RNA phenotype analysis based on production of cDNA, followed by enzymatic amplification of specific fragments with the polymerase chain reaction, we have examined simultaneous expression of growth factor transcripts in single or small numbers of preimplantation mouse embryos. Platelet-derived growth factor A chain (PDGF-A), transforming growth factor-α (TGF-α), and transforming growth factor-β (TFG-β) transcripts were found in whole blastocysts. TGF-α and PDGF antigens were detectable in both the inner cell mass and trophectoderm of the blastocysts by immunocytochemistry. Both PDGF-A and TGF-α were first detected as maternal message in the unfertilized ovulated egg, disappeared by the eight-cell stage, and then reappeared in early cavitation blastocysts. Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), nerve growth factor-β (NGF-β) and granulocyte colony stimulating factor (G-CSF) transcripts were not found in blastocysts. The expression of a unique subset of growth factors in mouse blastocysts suggests a role for these factors in the growth and differentiation of early mammalian embryos.

Introduction

Mouse preimplantation embryos grow and differentiate in the absence of exogenous factors, and thus, endogenous factors must sustain the embryo during the first six cleavage divisions (Biggers, 1971). These endogenous factors could constitute a clock similar to that which operates during early frog cleavage divisions (Newport and Kirschner, 1982a, b). However, unlike the abbreviated xenopus cleavage cell cycles, mouse preimplantation embryos have near-normal mammalian cell cycle times (Molls et al., 1983), and integrate information from extracellular sources. The paradigm for extracellular regulation of growth and differentiation is growth factor-growth factor receptor interaction.

A body of indirect evidence indicates that preimplantation embryos make growth factors. First, cultured preimplantation embryos produce transforming growth factor-like bioactivity that promotes anchorage-independent growth (Rizzino, 1985). It is not known if this activity is due to TGF-α, TGF-β, PDGF, or some unknown anchorage-independent growth-stimulating activity. Second, shortly after implantation in the uterus mouse embryos produce FGF protein in mouse (Risau, 1986, Risau and Ekblom, 1986), mouse embryos produce TGF-α protein and mRNA (Lee et al. 1985a), Twardzik, 1985), mouse embryos produce TGF-β protein (Heine et al, 1987), human embryos produce IGF-II transcripts (Scott et al., 1985) and mouse embryo produce IGF-II polypeptide (D'Ercole and Underwood, 1980), and *int-2* mRNA in mouse (Jacobovitz et al., 1986). These factors have been implicated only in the later phases of post-implantation growth and differentiation, and their presence may not indicate growth factor production by the preimplantation embryo. Other evidence for growth factor production in early mammalian embryogenesis comes from teratocarcinoma cells which are thought to be similar to the primitive ectoderm (Martin, 1975). The differentiated progeny of some teratocarcinoma lines are also equivalent to endodermal cells of the blastocyst (Adamson, 1986). Undifferentiated teratocarcinoma cells

produce PDGF protein (Van Veggel et al., 1987), and three protein stem cell growth factors (Jakobovitz et al., 1985). Differentiated teratocarcinoma cells also respond to NGF (Liesi et al., 1983), IGF-II (Heath and Shi, 1986), EGF (Adamson and Hogan, 1984) and PDGF (Rizzino and Bowen-Pope, 1985). Whether these transformed cells accurately depict preimplantation embryos is not known, because transformation may be caused by the improper expression of growth factors or receptors in these lines.

Direct evidence for growth factor transcripts in low copy number has been heretofore impossible to obtain in preimplantation embryos. Localization of mRNA transcripts in embryos by *in situ* hybridization is difficult (Han et al., 1987), and no data for growth factor transcripts have been published using preimplantation embryos. Thousands of embryos are required to detect high copy number transcripts such as histone or actin by RNA blotting analysis (Piko and Clegg, 1982; Giebelhaus et al., 1983). We report here a novel method for assaying, unambiguously and simultaneously, the accumulation of several growth factor transcripts in small numbers of mouse embryos.

Results

RNA Phenotyping Method for mRNA Analysis

We first developed a procedure that overcomes the difficulties inherent in the analysis of mRNA transcripts in preimplantation mouse embryos (Fig. 1). This procedure consists of a microadaptation of the GuSCN/CsCl technique for isolation of total RNA from 1-100 mouse embryos, coupled with two enzymatic steps. The purified RNA is reverse transcribed into cDNA in several cycles of reverse transcription using oligo-dT priming by standard techniques (Zeff, 1987). The products of this reaction are divided, and cDNA fragments specific for growth factors are amplified in a polymerase chain reaction (PCR) (Saiki et al., 1985) using the thermostable DNA polymerase from *Thermus aquaticus* (Taq) (Saiki et al., 1988). Each

Figure 1. Schematic representation of the steps involved in detecting the simultaneous mRNA phenotype in single embryos.

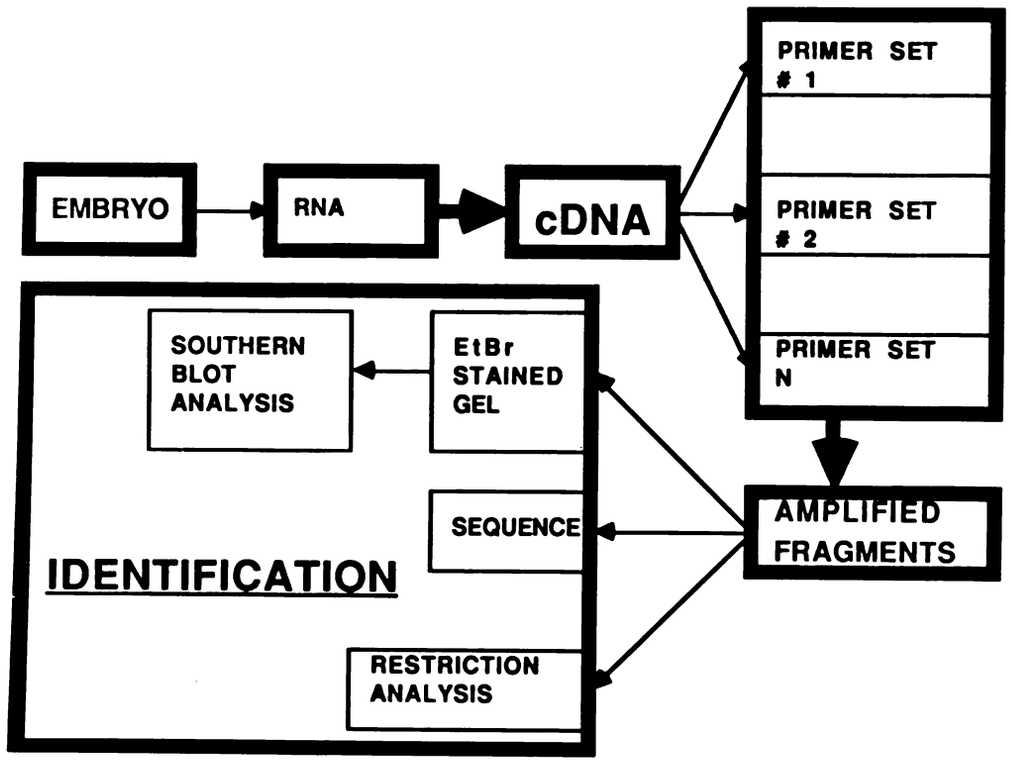

EMBRYO
RNA
cDNA
PRIMER SET # 1
PRIMER SET # 2
PRIMER SET N
AMPLIFIED FRAGMENTS
SOUTHERN BLOT ANALYSIS
EtBr STAINED GEL
SEQUENCE
RESTRICTION ANALYSIS
IDENTIFICATION

Figure 1a

chain reaction is primed by a pair of specific 20-25 base oligonucleotides that bracket a target sequence of 0.2-0.5 Kb, and that are chosen for (a) sequence specificity, (b) potential diagnostic traits such as restriction endonuclease sites, and (c) unique interaction with only the mature, processed RNA transcript. The structure of the target sequences for the growth factor transcripts used in this study are shown in Fig. 2. Oligonucleotides that cross intron-exon borders do not anneal with genomic DNA, or cDNA from unprocessed RNA under PCR conditions. Oligonucleotide pairs that generate fragments including introns would be predictably longer if DNA, or unprocessed RNA, contaminates the reaction. Where possible, fragments are selected to contain restriction sites, or to be identified by existing cDNA clones by Southern blot analysis. When mouse cDNA sequences were not available, heterospecific oligonucleotides were chosen to cover areas of conserved sequence with nondegenerate amino acids on the 3′ inside ends.

We first showed that we could detect β-actin RNA transcripts isolated from 2.2 mouse blastocysts using reverse transcription followed by 30 cycles of PCR, and that the signal became stronger by increasing the number of PCR cycles (Fig. 3a).

Preimplantation Mouse Blastocysts Express TGF-α, TGF-β and PDGF-A Transcripts

We then used reverse transcription followed by PCR to determine whether we could detect growth factor transcripts in blastocysts. Using RNA from the equivalent of 2.2 blastocysts we detected specific PCR products derived from TGF-α transcripts by 30 cycles of PCR (Fig. 3b). Next we determined the growth factor mRNA phenotype of blastocysts. We have found two other growth factor genes, TGF-β, and PDGF-A chain, are expressed in mouse blastocysts in addition to TGF-α (Fig. 4). Blastocysts were not cultured and therefore growth factor trancript expression is not induced during prolonged handling of embryos. The fragments so generated are identical to those produced from adult mouse tissues known to

Figure 2. (a) Schematic diagram of the four cDNA fragments generated from mouse embryo mRNA. Rectangles are 5′ and 3′ primers, triangles indicate introns, and vertical slashes indicate restriction endonuclease sites. Note that primers are actually on complementary fragment strands.

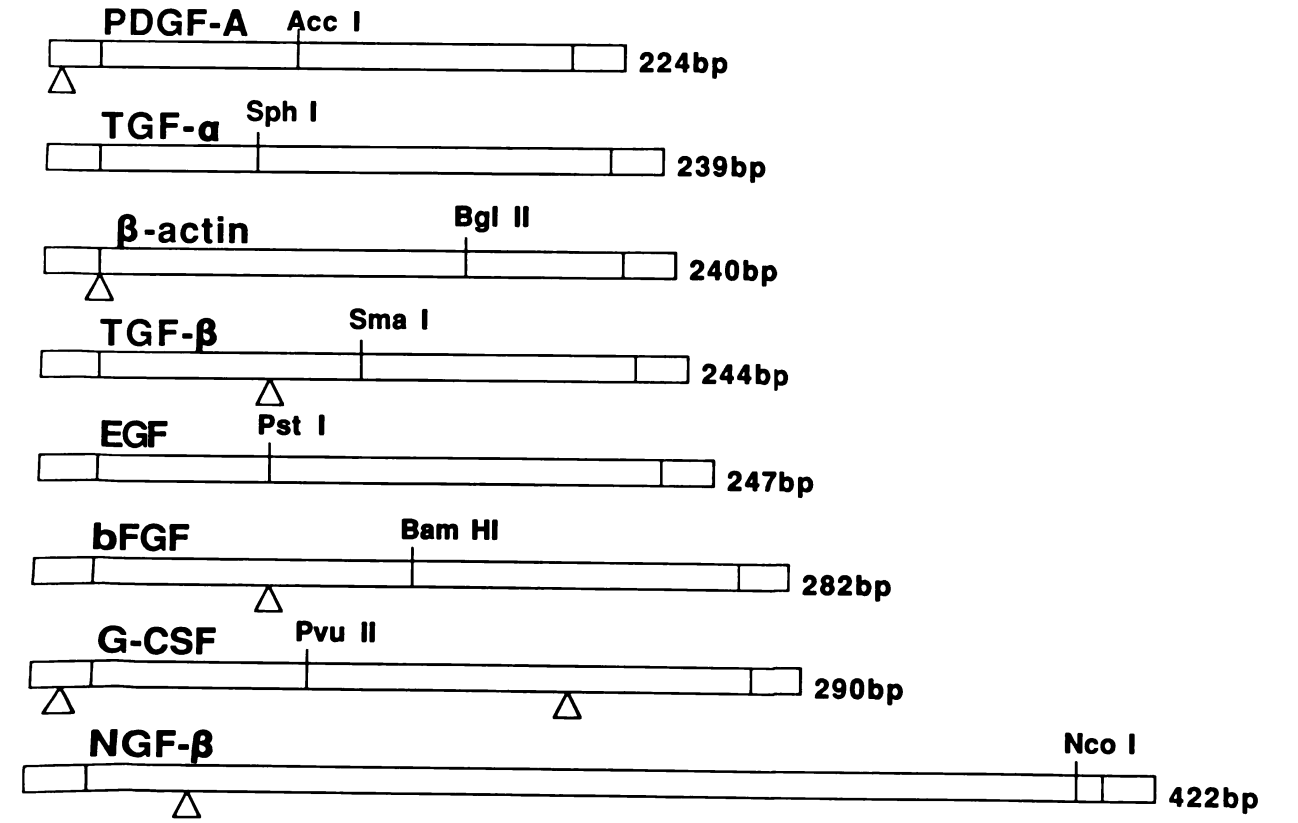
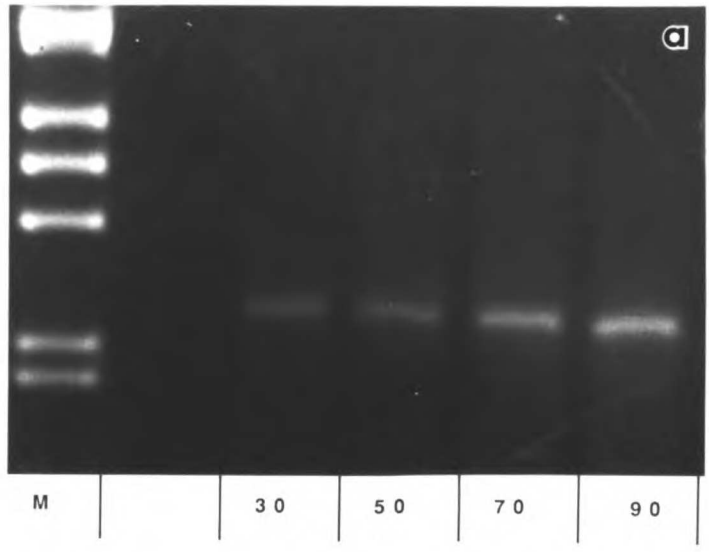

PDGF-A
Acc I
224bp
TGF-α
Sph I
239bp
β-actin
Bgl II
240bp
TGF-β
Sma I
244bp
EGF
Pst I
247bp
bFGF
Bam HI
282bp
G-CSF
Pvu II
290bp
NGF-β
Nco I
422bp

Figure 2

Figure 3. Demonstration of mRNA transcripts in mouse blastocysts by RT-PCR amplification after 30-90 cycles of PCR. (a) Actin transcripts from 2.2 blastocysts. (b) TGF-α transcripts from 2.2 blastocysts.


a
M
30
50
70
90
β-actin
Cycle number

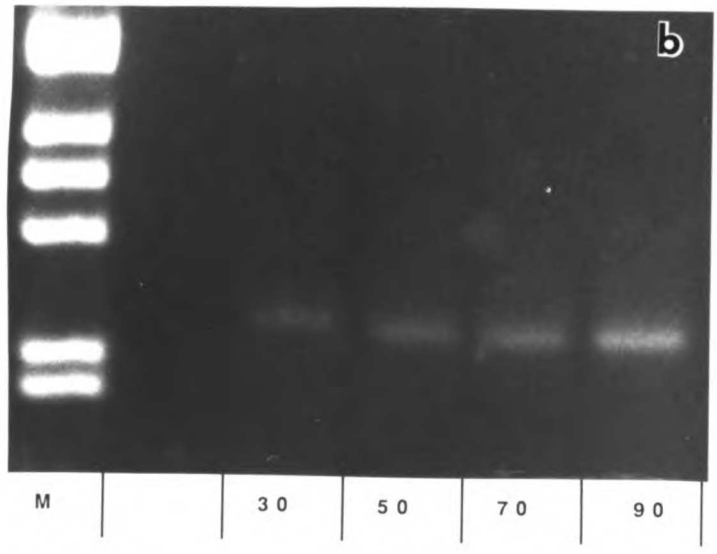

b
M
30
50
70
90
TGF-α
Cycle number

Figure 3

Figure 4. Mouse blastocysts express three growth factor transcripts. Agarose gel electrophoretic analysis of the RT-PCR amplified reaction products, obtained after 60 cycles of PCR. The PCR reaction mixtures for the blastocysts (B) contained cDNA from the RNA of 2.2 embryo equivalents in each reaction, and PCR reaction for the positive controls (+) contained cDNA from 10 ng RNA. The positive controls are brain for TGF-α, and macrophage for PDGF-A, TGF-β, and β-actin. The molecular size markers are Hae III-digested ϕX 174 replicative form DNA.

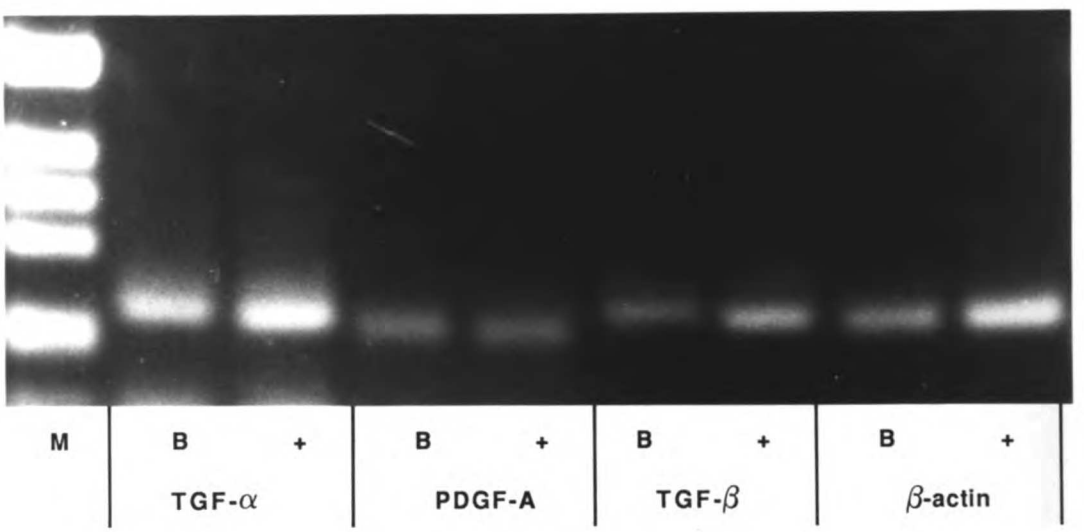


Figure 4

express these transcripts. Analysis of the restriction enzyme cleavage pattern of the fragments generated in the PCR was used to validate the identity of these fragments (Figs. 2 and 5). We also validated the identity of PCR reaction products by Southern DNA blot analysis (Fig. 5b), under hybridization stringency that requires more than probe-primer interaction to give a positive signal. Although the message complexity is lower in the blastocyst than unfertilized eggs (Schultz, 1986), it is necessary to rule out the possibility that blastocysts express growth factor genes promiscuously. Thus, we looked for, but did not find, evidence for expression of four other growth factors, EGF, bFGF, NGF-β and G-CSF, although these genes are expressed in tissues in adult mice (Fig. 6). The whole RNA dilution experiments indicated that detection threshold for these four transcripts in control cells was 10-10,000 less than the amount of whole RNA provided by the blastocyst assayed. These experiments suggest that mouse blastocysts initiate transcription of a select group of growth factors.

TGF-α and PDGF-A Transcripts are Also Present as Maternal Transcripts

The growth factor transcript accumulations are temporally controlled. PDGF-A is first detected as a maternal transcript in unfertilized ovulated oocyte (Fig. 7a). It disappears at the eight-cell stage, before reappearing in early cavitation blastocysts (32-64 cell stage). Control experiments suggest the difference in PDGF-A expression represents at least 10-100 fold increase in transcript numbers from 8 cell to blastocyst stage (Rappolee et al., 1988), but whether the absence of PDGF-A at the 8 cell stage is due to specific degradation is not known. TGF-α is also maternal transcript which disappears after fertilization and reappears before the blastocyst stage (Fig. 7b). As a control we used the transcript for the metalloproteinase, stromelysin (Frisch et al., 1987), which is present throughout preimplantation embryonic development (Brenner et al., manuscript in prep). Many growth factor transcripts carry an AUUUA 3′ untranslated end motif that confers instability on the transcript (Shaw and Camen, 1986, Brawerman, 1987). TGF-β transcripts appear only after

Figure 5. (a) Agarose gel electrophoretic analysis of the restriction endonuclease digestions of cDNA fragments generated by RT-PCR (60 cycles) from mRNA transcripts expressed by blastocysts reacted with the enzymes shown in Fig. 2. The diagnostic fragments shown are TGF-α, 159 bp TGF-β, 119+125 bp β-actin, 181 bp PDGF-A 129 bp. Restriction analysis of DNA fragments generated from RT-PCR. 40 μl of ethanol-precipitated RT-PCR fragments were digested with restriction enzymes as indicated in Fig. 2a, according to manufacturer's instruction (Bethesda Research Labs and Promega). Paired digested (Cut) and undigested (Uncut) fragments were fractionated by electrophoresis on a 4% agarose gel, and stained with ethidium bromide. The diagnostic fragments are: TGF-α, 159 bp; TGF-β, 125 + 119 bp; β-actin, 151 bp; PDGF-A, 129 bp.

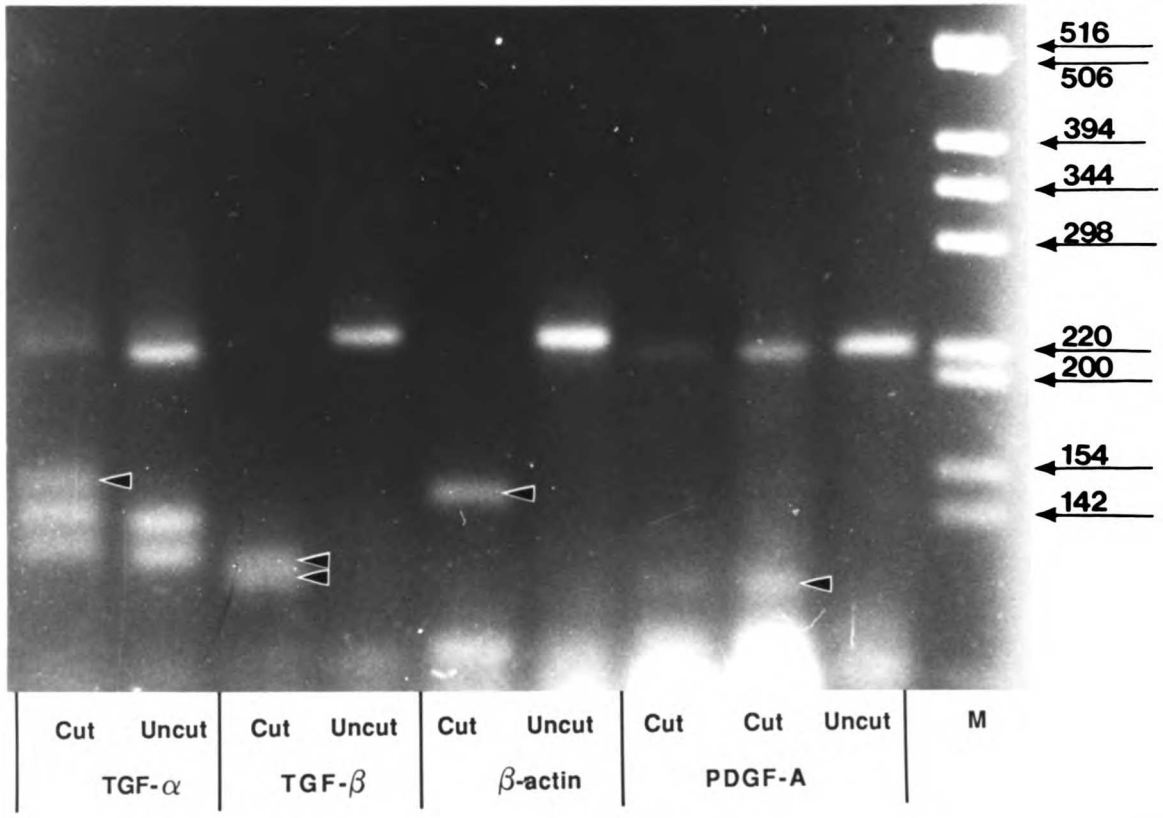

516
506
394
344
298
220
200
154
142
Cut
Uncut
Cut
Uncut
Cut
Uncut
Cut
Cut
Uncut
M
TGF-α
TGF-β
β-actin
PDGF-A

Figure 5a

Figure 5b. Southern analysis of RT-PCR products indicating TGF α expression in mouse blastocysts; M=1Kb ladder, 1, 4=TGF α, 2=TGF-β, 7=β-actin, 3=NGF, 5=FGF, 6=EGF. Upper panel; RT-PCR fragments (PCR was terminated at 60 cycles) visualized in 4% agarose by ethidium bromide. Lower panel; Southern blot analysis of products transferred from the gel shown in the upper panel. The gel was transferred to a nylon membrane (Reed and Mann, 1987) and hybridized with random primed TGF-α cDNA (cDNA was a gift of R. Derynck) and washed 1xSSC, 0.2% SDS, 65°C, 1 hr.

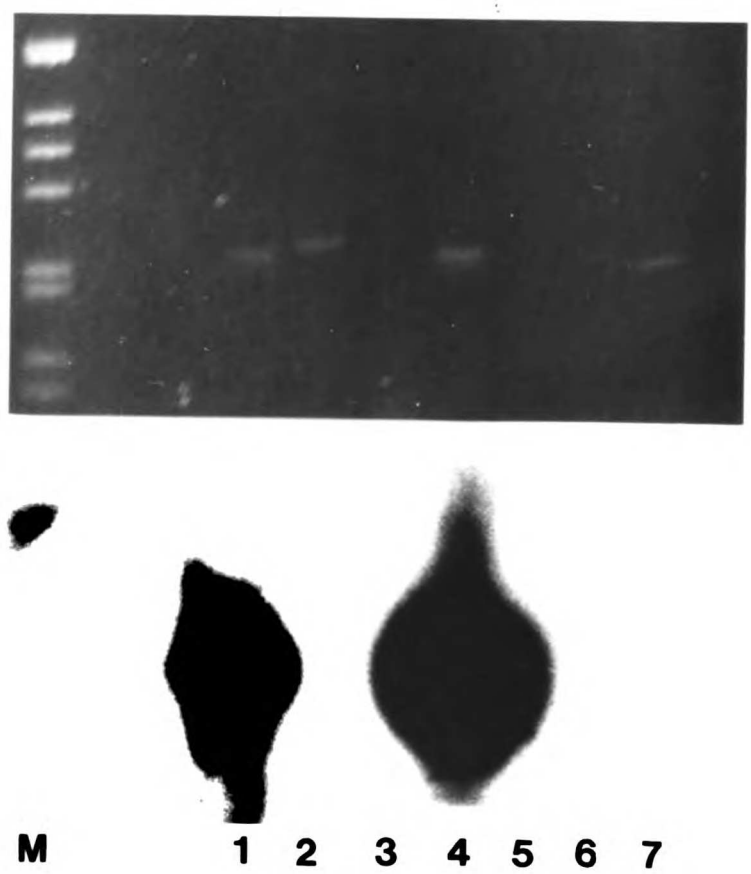


Figure 5b

Figure 6. Mouse blastocyst does not express four other growth factors. Agarose gels electrophoretic analysis of the RT-PCR amplified reaction products obtained after 60 cycles of PCR. The PCR mixtures for blastocysts (B) contained cDNA prepared from mRNA of 2.2 embryo equivalents in each reaction, and for the positive controls (+) from 10-100 ng of total RNA from submaxillary gland for EGF, brain for bFGF, fibroblasts for NGF-β, and endotoxin-stimulated macrophages for G-CSF. Predicted sizes of amplified fragments are: EGF=350 bp, bFGF=282 bp, NGF-β=422 bp, and G-CSF=294 bp.

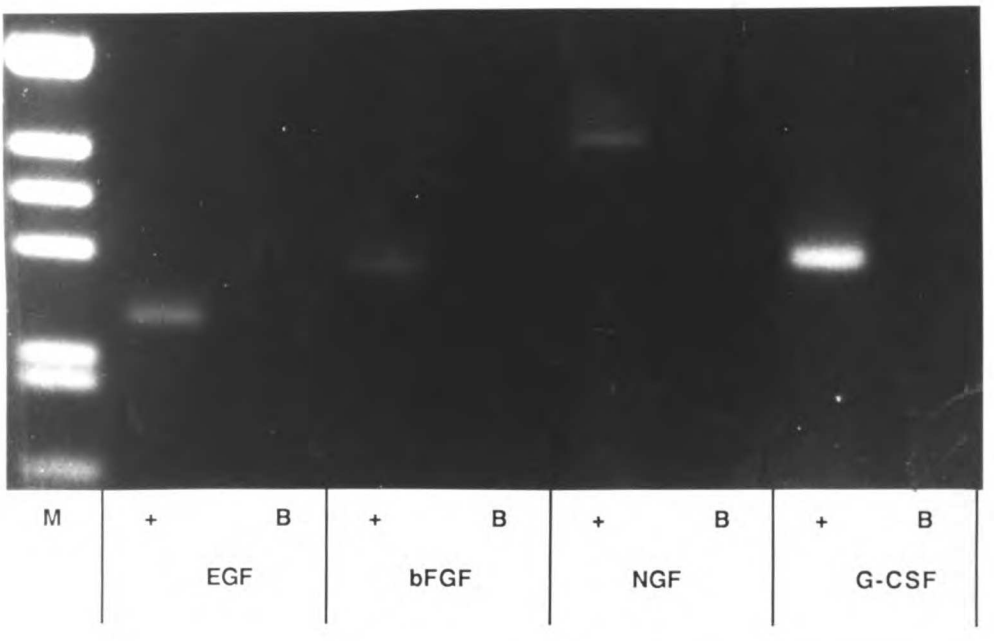


Figure 6

Figure 7a. PDGF-A expression is temporally regulated in the preimplantation mouse embryo. Agarose gel electrophoretic analysis of RT-PCR amplified reaction products obtained after 60 cycles of PCR. The PCR mixtures each contained cDNA prepared from the equivalent of the mRNA of 4.2 unfertilized ovulated oocytes (Egg), 6.8 8-cell zygotes (8-cell) or 3.6 early cavitation (32-cell) blastocysts (B). The positive control for the RT-PCR reactions are the amplified fragments 212 bp from transcripts of the proteinase, stromelysin, which is present in all the embryonic stages shown (Brenner et al., manuscript in preparation).

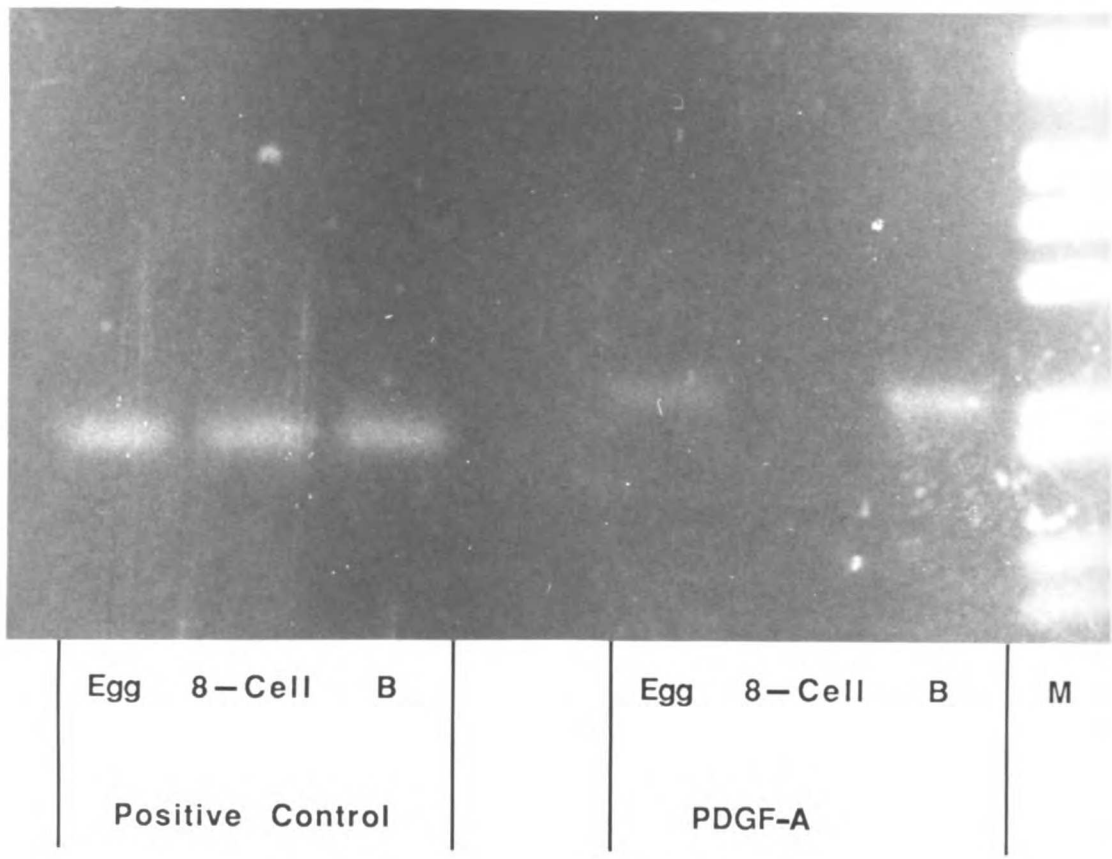
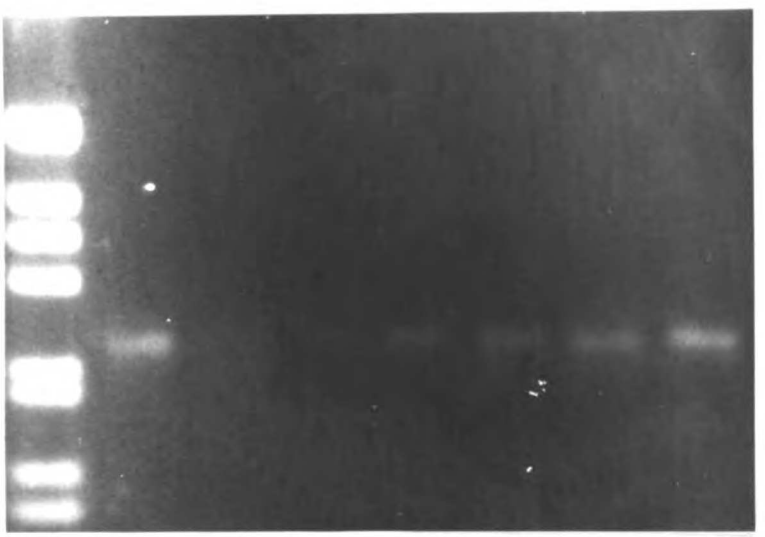


Figure 7a

Figure 7b. TGF-α expression is temporally regulated in the preimplantation mouse embryo. Agarose gel electrophoretic analysis of RT-PCR amplified reaction products obtained after 60 cycles of PCR. PCR mixtures contained cDNA prepared from the equivalent of the mRNA of 4 unfertilized ovulated oocytes (E), 8.8 2-cell embryos (2), 5.2 4-cell embryos (4), 2.4 16-cell embryos, 4.4 blastocysts (B) or 1 ng of RNA from a blastocyst cell-like Nulli (N) cell line.



TGF-α in mouse embryos

(E=unfertilized egg; 2,4,8,16=#cells; B=blastocyst; Nulli=teratocarcinoma cell line)

Figure 7c

activation of the zygotic genome at the 8 cell stage, unlike TGF-α and PDGF A transcripts (data not shown).

Mouse Blastocysts Express TGF-α and PDGF Antigens

Transcription of growth factor mRNA is not invariably coupled with the translation of these transcripts into protein. We sought to determine whether mouse blastocysts also translate these transcripts. Because the efficiency of existing polyclonal antisera to TGF-α and PDGF precluded their use for immunoprecipitation of radiolabeled mouse egg and blastocyst lysates, and conditioned medium, we identified TGF-α (Fig. 8) and PDGF (Fig. 9) antigens by immunocytochemical localization in permeabilized blastocysts. The immunofluorescent signal concentrated in the perinuclear area was present in all cells of the blastocyst for both antigens. This pattern indicates the cells are probably synthesizing the growth factor. The signal was diminished by initially incubating the antisera with purified soluble antigens, indicating that the embryonic antigens react with specific epitopes in the growth factor amino acid sequence. The high nonspecific background in unfertilized oocytes precluded the determination, by immunofluorescence, of expression of these growth factor antigens.

Discussion

The relatively autonomous behavior of mammalian embryos before implantation suggests that their growth and differentiation may be sustained by endogenous growth factors. Using a novel technique for RNA phenotyping, we have provided direct evidence that TGF-α, TGF-β and PDGF-A genes are expressed in mouse blastocysts. We find that accumulations of PDGF-A transcripts are found in unfertilized, ovulated oocytes, disappear by the eight-cell stage, and zygotic PDGF-A reappears at the early blastocyst stage. TGF-α was also seen from unfertilized oocyte to blastocyst. Although, growth factor transcription can take place

Figure 8. TGF-α antigen is expressed in mouse blastocysts. a and b are phase and immunofluorescence micrographs of blastocysts reacted with anti-TGF α, C and D are phase immunofluorescence and phase blastocyst by immunocytochemistry. micrographs of blastocysts stained with anti-TGF-α absorbed with TGF-α antigen, and e and f are normal rabbit serum controls.

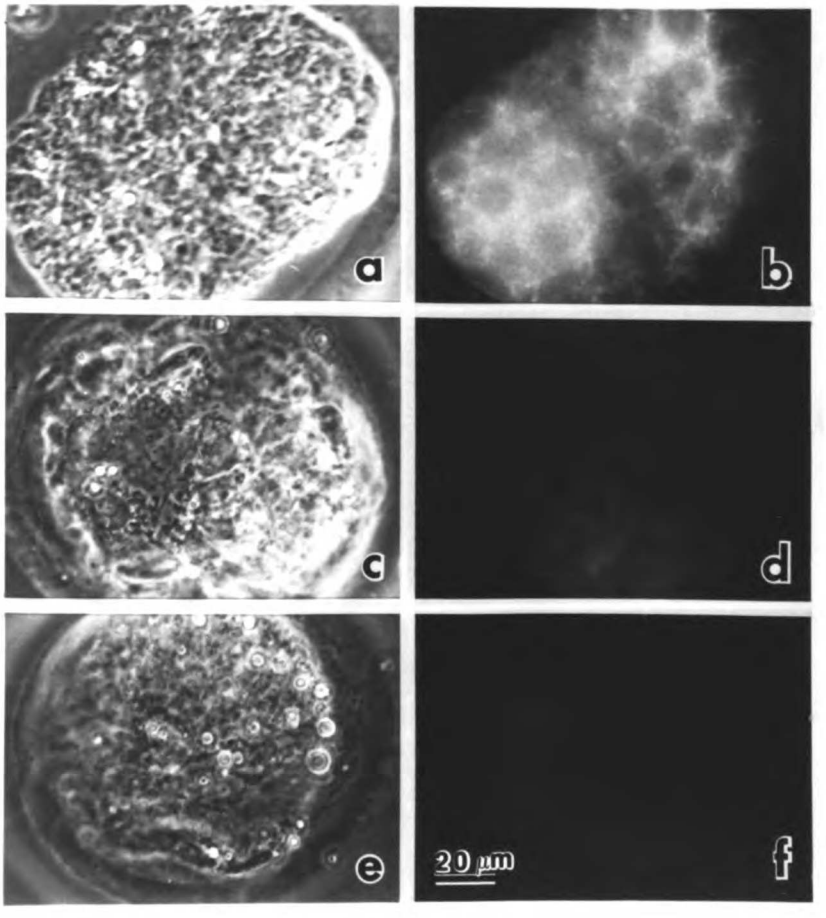


Figure 8

Figure 9. PDGF antigen is expressed in mouse blastocysts. a, a^1 and b, b^1 are phase and immunofluorescence micrographs at two different focal planes of a mouse blastocyst with stained anti-PDGF; c and d are phase and immunofluorescence micrographs of a blastocyst stained with normal goat serum. The arrow in b^1 shows a mural trophectoderm cell.

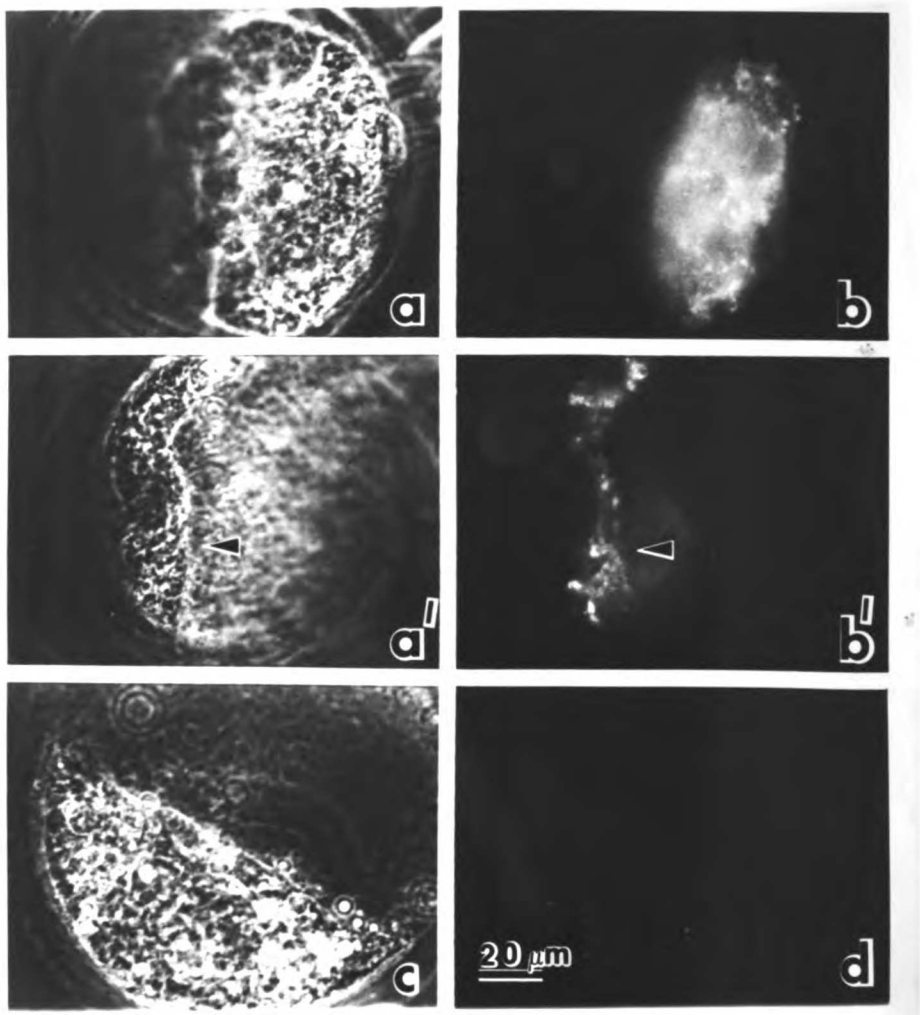


Figure 9

without translation (Assoian et al., 1987), this did not occur in blastocysts where we detected TGF-α and PDGF antigens in the blastocyst by immunocytochemistry.

Single Cell mRNA Phenotyping

We have developed a new method for analyzing the mRNA phenotype of preimplantation mouse embryos. The combination of reverse transcription followed by polymerase chain reaction allows detection of < 100 transcripts of cRNA and transcripts from a single cell (Rappolee et al., 1988). In addition the products of the reverse transcription can be divided and primed separately in the PCR, a process we call "single cell mRNA phenotyping". This method has been used to detect growth factor transcripts in small numbers of pre-implantation embryos where polypeptide growth factor transcripts have previously been undetected. Thus, this method provides a powerful new approach for determining the mRNA phenotype of rare tissues and cells at early stages of embryonic development, before the onset of terminal differentiation, and when only the most abundant transcription products can be detected by other, less sensitive methods.

Production of amplified fragments in the PCR is approximately described by a derivation of the equation for bacterial growth, $N=N_0E^n$, where E is the efficiency of the reaction (which has a range of 1 to 2, that corresponds to a doubling efficiency of 0-100%, for 50% efficiency E=1.5), n is PCR cycle number, N_0 equals input cDNA, and N equals final number of fragments. Using this equation and the results from a single cell experiment (Rappolee et al, 1988), we estimate that each cDNA can produce 10^9 fragments and that the PCR average efficiency for cDNA is 35-55%. From this equation it is evident that small changes in PCR efficiency produce large changes in N. We are able to resolve three-fold differences in input RNA, and, therefore, threefold differences in RNA between internally controlled samples. Reverse transcription followed by PCR can therefore provide a means of rapid qualitative transcriptional phenotyping of low cell or transcript numbers. The

quantitation of each transcript requires an internal stand curve generated from cRNA that has a sequence identical to the endogenous mRNA over the range from the 5′ primer to the poly-A tail. The technique also allows rapid cross-species cloning and sequencing and production of high specific activity DNA probes (Rappolee et al., 1988).

Embryonic Growth Factors

The mouse embryo grows autonomously for only the first 7-8 cell divisions at which time it interacts with, and implants in the wall of the uterus. The controlling influences on these first divisions have not been studied. Zygotic gene transcription begins after the first cell division. To date, there has been no direct proof that preimplantation mouse embryos synthesize growth factors, although there are indirect results suggesting that embryos can bind and express specific growth factors. For example, preimplantation mouse embryos specifically bind EGF (Adamson, 1986) and peri-implantation mouse embyros cultured for 2 days produce transforming growth factor-like biological activity (Rizzino, 1985). In this report, we have provided direct evidence that TGF-α, TGF-β, and PDGF-A genes are expressed in mouse blastocysts, and that this transcription is selective; blastocysts do not transcribe genes for EGF, bFGF, NGF-β or G-CSF. It is likely that TGF-α, PDGF, and TGF-β described here, alone or in combination, account for the transforming growth factor-like activity described by Rizzino (1985).

On the other hand, there is ample evidence for growth factor production and/or responsiveness in post-implantation fetal development. First, post-implantation rodent embryos at 7.5 days of later stages of gestation produce and/or respond to TGF-β (Heine, 1987), NGF (Levi-Montalcini and Booker, 1960), TGF-α (Lee et al., 1985a, Twardzik, 1985), FGF (Risau, 1986; Risau and Ekblom, 1986), IGF-II (Adamson, 1986, D'Ercole and Underwood, 1980) and *int-2*, transcript, which has a sequence related to FGF (Jacobovitz et al., 1986). Although most growth factors are not expressed in mouse embryos until the

organogenesis phase (day 9), *int-2* (Jacobovitz et al., 1986) and TGF-α (Twardzik, 1985) are expressed by 7.5 days, only two days after implantation. Second, embryonal carcinoma stem cell lines which resemble the pluripotential cells of the inner cell mass, in the preimplantation embryo, and their immediate progeny of endodermal lineage produce NGF-β (Liesi et al., 1983, Dicou et al., 1986), PDGF (Rizzino and Bowen-Pope 1985), FGF (van Veggel et al., 1987), and TGF-α (Heath and Rees, 1985). However, transformation of these stem cell lines may involve irregular growth factor receptor or ligand expression. We find no NGF-β expression in blastocysts whereas a trace of NGF-β has been found in F9 and PCC4 embryonal carcinoma cell lines (Dicou et al., 1986). Third, embryonal carcinoma stem cells and/or their progeny specifically bind EGF (Adamson and Hogan 1984), IGF-II (Heath and Rees, 1986), PDGF (Rizzino and Bowen-Pope, 1985) and NGF (Liesi et al., 1983). Taken together these findings extrapolate back to a potential for function of growth factors in early embryonic growth development in mammals.

Growth factors have been implicated in the embryonic development of diverse non-mammalian species. FGF and TGF-β appear to be morphogens for inducing mesoderm at the blastulation stage in Xenopus (Slack et al., 1987; Weeks and Melton, 1987; Kimelman and Kirschner, 1987, Rosa et al., 1988), TGF-β-like and EGF-like molecules may influence *Drosophila* development (Padgett et al., 1987, Wharton et al., 1985, Hafen et al., 1987) EGF-like molecules may influence nematode development (Greenwald et al., 1985), and IGF-II-like and FGF-like molecules may influence chick development (Bell, 1986; Goldin and Opperman, 1980, S. Hauschka et al., personal communication). These growth factors can induce differentiation as in Xenopus (Kimelman and Kirschner, 1987; Slack et al., 1982, Rosa et al., 1988), or induce both differentiation and mitosis as in chick (Bell, 1987; Goldin et al., 1980). In the frog, growth factors influence differentiation at cell division number eight (Kimelman and Kirschner, 1987). In the mouse we find maternal growth factor transcripts

replaced by zygotic growth factor transcripts before the sixth cell division. Early development in mouse has several other properties which distinguish it from that of frog. First, the egg is small, has little yolk, and quickly activates its zygotic transcription after fertilization (Schultz, 1986). Second, the mouse has 10-to-12-hour cell cycle times (Molls et al., 1983; Pedersen, 1986) after the first two cell cycles. These cycles have the normal G1/S/G2/M periods, in contrast with the early cell divisions of frog which lack G1 and G2 (Graham and Morgan, 1966). The presence of G1 and G2 in cleavage stage mouse embryos may allow transcription of growth factors, as well as the opportunity to be influenced by growth factors.

The accumulation patterns of growth factor transcripts in preimplantation mouse embryos fall into two classes. In one class, including PDGF-A and TGF-α in mouse, maternal transcripts apparently disappear and are resynthesized in the zygote. A similar situation occurs for frog FGF (Kimelman et al., 1987), and PDGF-A (C. Stiles, personal communication). In the other class, transcripts survive the breakdown of maternal mRNA which is initiated during meiotic maturation, becomes quite dramatic in the 2-call embryo, and continues up to the blastocyst stage (Schultz, 1986). TGF-β is an example of the second class. Similarly, in Xenopus, the TGF-β-like Vg-1 which is localized to the vegetal hemisphere and may play a role in mesoderm induction, persists throughout early development (Weeks and Melton, 1987). A comparable physiological role for TGF-β in mouse is not known.

What does the presence of these three early growth factor transcripts imply about their function in mouse embryos? We can divide the growth factor function using two criteria: direction and action. The direction can be within the embryo or between the embryo and the mother. The action can be to influence mitosis and/or differentiation. An intraembryonic mitogenic function is suggested by the coincidental production, by the autonomous blastocyst of the three growth factors belonging to a factor subset that sustains anchorage-independent growth (Anzano et al., 1987). The onset of growth factor transcription from the zygotic

genome in mouse roughly coincides with, or precedes, the differentiation of totipotent inner cell mass cells into primitive ectoderm and endoderm. Several lines of evidence indicate that embryonic factors are directed at maternal tissue. The strongest evidence is the prolongation of corpus luteum lifespan in sheep by an embryonic protein thought to be ovine trophoblast protein-1 (Weitlauf, 1988). This protein, which binds endometrial receptors and is the major translation product of ovine trophoblasts, was recently cloned and found to be highly homologous to a secreted polypeptide factor, α_{11}-interferon, (Imakawa et al., 1987). Our evidence for embryonic-maternal communication is more circumstantial. First, TGF-α and TGF-β are known to be angiogenic (Schreiber et al., 1985; Roberts et al., 1986), and the highest density of uterine capillary beds are opposite the implanting blastocyst (Williams et al., 1948). In addition, the uterine environment is hypoxic (Yochim, 1971), a condition that promotes wound healing cells to produce angiogenic factors (Knighton et al., 1983). Finally, at the time of implantation there is a surge of estrogen that increases EGF receptor expression in uterus several-fold (Mukku and Stancel, 1985) and TGF-α is an EGF receptor-binding ligand. Taken together these data indicate that embryonic growth factors may induce the early angiogenesis and decidualization of the uterus. Our sensitive technique for determining mRNA phenotype in a single or several embryos will allow testing of these hypotheses.

Experimental Procedures

Materials

Taq polymerase was obtained from Perkin Elmer Cetus and polymerase chain reaction was performed using a programmable DNA Thermocycler heating block from Perkin-Elmer Cetus. Restriction enzymes were obtained from New England Biolabs and Bethesda Research Labs. Radioisotopes were purchased from Amersham. MMLV reverse

transcriptase was purchased from BRL. Mice were purchased from Jackson Labs and Charles River. Rabbit antibodies prepared against synthetic TGF-α antigen, and synthetic TGF-α were purchased from Peninsula Labs. Goat anti-human PDGF antibodies were a kind gift of G. Grotendorst. Human PDGF was obtained from Collaborative Research. Affinity purified biotinylated anti-rabbit IgG and anti-goat IgG antibodies were purchased from Sigma. Texas-Red-labeled streptavidin was purchased from Amersham. Primers for reverse transcription were obtained from Pharmacia. Oligonucleotide primers for the polymerase chain reaction were obtained from the U.C.S.F. Biomolecular Resource Center. cDNA clones for TGF-α and TGF-β were gifts of R. Derynck, Genentech, Inc., and for PDGF-A from Christer Betsholtz (Betsholtz et al., 1986).

Mouse Eggs and Embryos

Standard techniques were used for obtaining eggs and zygotes (Hogan et al., 1986). Briefly, female CF-1 mice (25-30 gm, Harlan Sprague-Dawley) were injected intraperitoneally with 10 IU of pregnant mare mares' serum (PMS, Sigma). An injection of 5 IU of human chorionic gonadotropin (hCG, Organon, Inc.) was administered 48-50 hr later. Females were mated overnight with stud F1 hybrid males (from C57B1/6J females x SJL/J males, Jackson Labs.). Successful mating was determined by presence of vaginal plugs. All embryonic ages were expressed as days of gestation (d.g.), designating noon of the plug day as 0.5 d.g. Ovulated unfertilized oocytes surrounded by cumulus cells, were dissected from oviducts 14 hr post-hCG. The egg mass was incubated in 1,000 μg/ml hyaluronidase (Sigma) to remove adherent cumulus cells. After digestion the eggs were aspirated away from cumulus. Cleavage stage embryos were flushed from the oviducts at 2.3 d.g., and blastocysts were flushed from the uteri at 3.5 d.g. with L-15 (Microbiological Associates) containing 4 mg/ml bovine serum albumin. The embryos or eggs were washed extensively to free them of any cumulus cells or

somatic cells fro oviduct or uterine flushing, then transferred with the use of mouth-operated micropipets under a dissecting microscope to insure that there was no somatic cell contamination.

RNA Preparation and Reverse Transcription.

A microadaptation of the GuSCN/CsCl procedures (Chirgwin et al., 1979) was used to prepare total RNA from embryos. One to several hundred eggs or embryos were washed through six drops of L-15 and added to 100 μl of guanidine thiocyanate solution containing 20 μg E. coli rRNA carrier were layered over 100 μl of 5.7 M CsCl and centrifuged for 20 x 10^6 *g*-min/cm gradient in the TLA-100 rotor of a Beckman TL-100 bench top centrifuge. Yieldsw of RNA were determined by reading $^{A}260$, and were generally 35-50%. Total RNA used for positive controls was prepared by the standard GuSCN/CsCl procedure (Chirgwin et al., 1979), from the following sources. bFGF and TGF-α, mouse brain, NGF-β, BALB/c 3T3 fibroblasts; TGF-β, β-actin, G-CSF and PDGF-A, lipopolysaccharide-stimulated mouse macrophage.

Pelleted RNA was washed with 80% ethanol, dried and reverse transcribed with oligo dT priming under conditions given by the manufacturer (Gerard, 1987). Reverse transcription was repeated by denaturing at 93°, flash cooling on ice and reincubating with an additional 50U of enzyme.

Polymerase chain reaction

PCR was performed essentially as previously described (Saiki et al, 1985, 1988). A small portion of RT products (1 μg) was mixed with 1 U of Taq DNA polymerase, 50 pmol of 20-25 base-long oligonucleotide 5′ and 3′ sequence-specific primers, in a buffer containing 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 50 mM KCl, 5 μg acetylated bovine serum albumin, pH 8.3, in 50 μl volume. The mixture was overlaid with mineral oil to prevent evaporation and then amplified

by PCR in a repeated 3-temperature cycle on the Thermocycler programmable heating block. For samples to be amplified for >60 cycles of PCR, 1 U of Taq polymerase was added at 60 cycles. Experiments were performed at least three times with independent embryo preparations.

Oligonucleotides

Primers were based on the following designs:

β-actin, mouse (Alonso et al., 1985)	5′ primer (5′-3′) GTGGGCCGCTCTAGGCACCA
	3′ primer (5′-3′) GGGGGACGTGGGATTCCGGT
TGF-α, mouse (Lee et al., 1985b)	5′ primer (5′-3′) ACCTGCAGGTTTTTGGTGCA
	3′ primer (5′-3′) CAGACGAGGGCACGGCACC
TGF-β, mouse (Derynck et al., 1986)	5′ primer (5′-3′) AAGTGGATCCACGAGCCCAA
	3′ primer (5′-3′) ACGCGAGGACGTTCACGTCG
PDGF-A, human (Betsholtz et al.,1986)	5′ primer (5′-3′) CCCCTGCCCATTCGGAGGAAG
	3′ primer (5′-3′) GTGGCGTCGCAGTTCCACCGT
EGF, mouse (Gray et al., 1983, Bell, 1986)	5′ primer (5′-3′) CCAGTTCAGTAGAAACTG
	3′ primer (5′-3′) CCTCTTTTAGTAATCTTTGGT
bFGF, human/bovine (Abraham et al., 1986a,b)	5′ primer (5′-3′) TACAACTTCAAGCAGAAGAG
	3′ primer (5′-3′) GTTTACAGACGATTCTCGAC
NGF-β, mouse (Scott et al., 1983)	5′ primer (5′-3′) CCAAGGACGCAGCTTTCTAT
	3′ primer (5′-3′) AAGTTGTCCTGAGTGGCCTC
G-CSF, mouse (Tsuchiya et al., 1986)	5′ primer (5′-3′) GCCCTGCAGCAGACACAGTG
	3′ primer (5′-3′) GTCCCGAAGGACCTCTGCCG

Stromelysin rabbit	
(Frisch et al., 1987;	5′ primer (5′-3′) CTGAGGTTTGATGAGAAGA
Wilhelm et al., 1987;	3′ primer (5′-3′) ACTTCTCGTTGTCGACCAAA
Werb unpublished)	
HSP-70	5′ primer (5′-3′) ACGACAAGGGCCGCCTGAGC
	3′ primer (5′-3′) GGCCTCTGCTGGCTCTCCCG

Indirect Immunofluorescence

For immunofluorescence embryos were freshly isolated from superovulated CF-1 mice at the blastocyst stage (3.5 d post coitum), or isolated at the two-cell stage (1.5 d.g.) then cultured for 2 days until they developed to the blastocyst stage. The embryos were placed in PBS containing 10% polyvinylpyrollidone (80 kDa), cytocentrifuged onto poly-L-lysine-treated 12-mm glass coverslips, then fixed in freshly prepared 2% paraformaldehyde for 30 min at room temperature. The coverslips were washed with 0.1 M glycine in PBS, permeabilized in 0.25% Triton X-100 in PBS for 4 min, and then treated for 30 min with 1 mg/ml ovalbumin in PBS. Non-specific binding sites were blocked with 5% normal sheep serum in PBS for anti-TGF-α staining, or 5% normal rabbit serum for anti-PDGF staining. The embryos were then treated with primary antibody diluted 1:100 in PBS (30 μl/coverslip) for 1 h at room temperature, washed with PBS containing 0.1% Tween 20, then incubated with biotinylated sheep anti-rabbit IgG for anti-TGF-α, or biotinylated rabbit anti-goat IgG for anti-PDGF at 1:100 dilution. Finally the embryos were stained with Texas Red-labeled streptavidin at 1:100. Immunofluorescence was photographed on Tri-x film rated at 800 ASA with a Zeiss Photomicroscope III, using a 63 X Plan Neofluor-phase water immersion lens. Exposures were for 60-120 sec. Controls included no first antibody, no second antibody, absorption of the anti-TGF-α antibody with TGF-α antigen (100 ng TGF-α to 0.5 μl antiserum), and

absorption of the anti-PDGF-antibody with human PDGF antigen (20 units PDGF to 1 μl antiserum).

DNA Blot Analysis

DNA separated on agarose gels was transferred to Zeta-bind nylon membranes as previously described (Reed and Mann, 1985). Gels were incubated for two 10-min periods at ambient temperature with 0.25 M HCl, incubated overnight in 0.4 M NaOH, and rinsed in 2x SSC (1x SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). cDNA probes were prepared by random priming (Maniatis et al., 1983). Zeta-bind transfers were hybridized by standard techniques (Maniatis, et al., 1982) and washed at 60°C for 60 min, in 0.2x SSC containing 0.1% SDS before autoradiography.

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Appendix B

Megakaryocyte α-granules contain exogenously and endogenously synthesized proteins.

Megakaryocytes are large polyploid cells which arise from pluripotent stem cells in bone marrow. Each mature megakaryocyte fragments into several thousand platelets. During maturation megakaryocytes synthesize 4 types of granules: lysosomes, peroxisomes, dense granules, and α granules. α granules contain hemostatic, inflammatory, and wound healing proteins which are released by platelets upon activation by thrombin (Bainton, 1980). Some of the α granule contents, notably platelet factor 4 (PF 4) and β-thromboglobulin, are thought to be exclusively limited to platelets (Doi *et al.*, 1987). A second class of α granule proteins, represented by albumin and immunoglobulin, are synthesized elsewhere in large quantity and are present at high concentration in blood (Handagama *et al.*, 1988). A third class of α granule proteins, represented by PDGF, is synthesized in many loci, but is in low concentration in blood. How then do these various proteins enter the α granules? PF 4 and β-thromboglobulin are believed to be transcribed and translated by megakaryocytes. It is possible that other proteins such as albumin or fibrinogen may be taken up by megakaryocytes entirely from the blood. It was recently demonstrated that horseradish peroxidase (HRP) can be taken up into megakaryocyte α granules from the blood; the endocytosed HRP was released along with other α granule contents upon thrombin activation of circulating platelets (Handagama et al., 1987). Albumin has a profile of megakaryocyte uptake and platelet release comparable to HRP (Handagama *et al.*, 1988). Proteins might also enter α granules of circulating platelets although such a phenomenon has not been observed in vitro (Zucker-Franklin et al., 1981).

We wished to test whether certain megakaryocyte α granule proteins are derived entirely from exogenous endocrine sources. In particular, fibrinogen and albumin which are made in large quantity by hepatocytes, seemed like good candidates for uptake. We expected that, in the case where fibrinogen and albumin were entirely derived from plasma, these proteins would be found in megakaryocytes, but their mRNA would not.

Figure 1. Primer structures and structures of amplified sequences. All primers are between 20 and 24 bp in length. Triangles indicate introns, vertical slashes indicate restriction sites and terminal boxes indicate 5′ and 3′ oligonucleotide primers. References for primer sequences; PDGF A, TGF-α, β-actin, and EGF were given in Chapter 3, PF4 (Doi et al., 1987), γ-fibrinogen (Crabtree and Kant, 1982), albumin (Sargent et al., 1981), and α-fibrinogen (Crabtree et al., 1985).

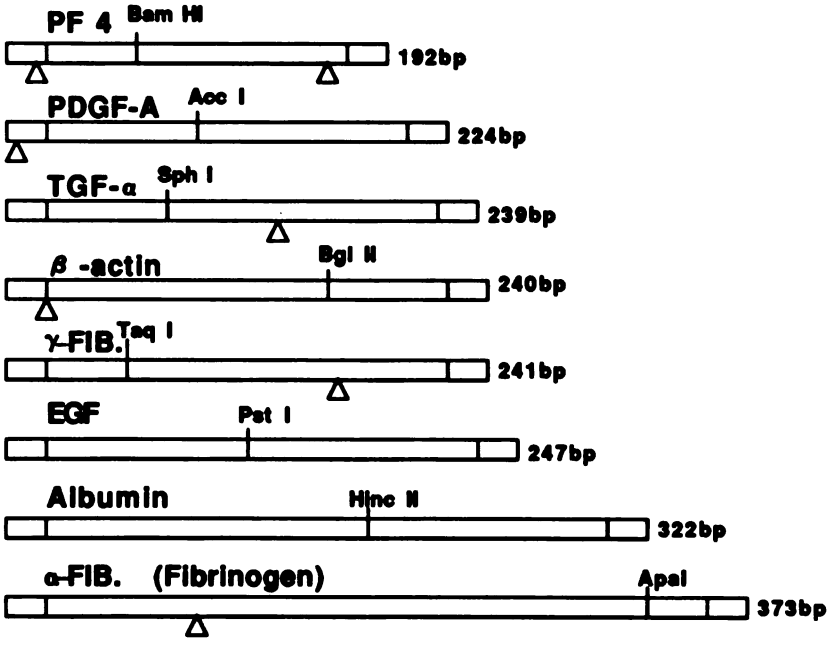

PF 4
Bam HI
192bp
PDGF-A
Acc I
224bp
TGF-α
Sph I
239bp
β -actin
Bgl II
240bp
γ-FIB.
Taq I
241bp
EGF
Pst I
247bp
Albumin
Hinc II
322bp
α-FIB. (Fibrinogen)
ApaI
373bp

Figure 1

Figure 2. Rat megakaryocytes transcribe TGF-α, but not α-fibrinogen. RT-PCR products (60 cycles of PCR) derived from the RNA from 1,000 megakaryocytes/lane were electrophoresed on 4% agarose and ethidium bromide stained. M=1 kb ladder, (see Chapter 2, figure 4, for sizes of markers) α-fibrinogen was not detected in lanes 1-4, TGF-α was detected in lanes 5-8. Each lane represents RNA purified separately from a single cellular prep. This experiment has been repeated, with similar result, 3 times. Bands were validated by size and by restriction analysis.

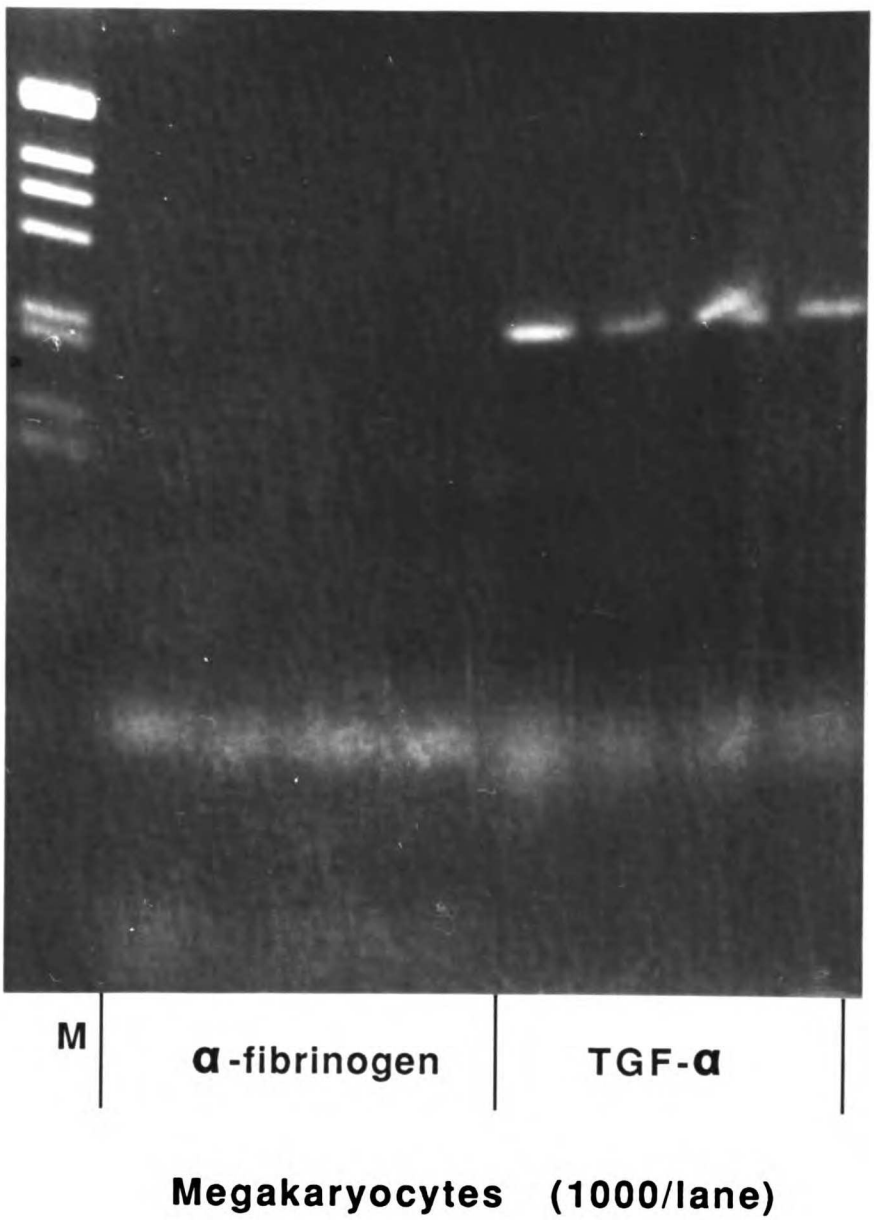


Figure 2

Figure 3. Detection threshold for α-fibrinogen is 100 hepatocytes. Whole liver RNA was serially diluted, and added to 1 μg E. coli rRNA, reverse transcribed and 1/10th of RT reaction was amplified using α-fibrinogen oligonucleotide primers by PCR (60 cycles of PCR). 10 pg was estimated to be the amount of RNA from rat one hepatocyte. Bands were validated as α fibrinogen by size and by restriction analysis.

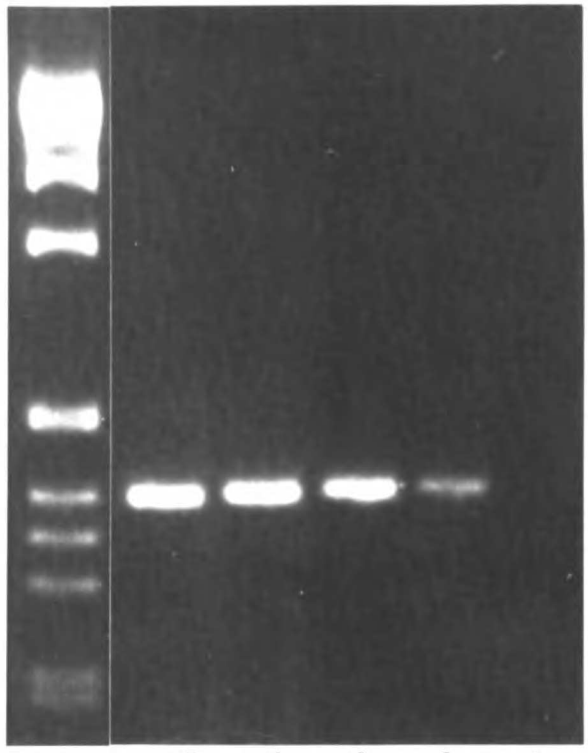


Figure 3

Figure 4. Guinea pig megakaryocytes contain fibrinogen protein. Whole bone marrow was prepared as described by Beckstead (1986). 1/500 rabbit anti-fibrinogen diluted 1/500 (Cappel), and HRP-conjugated anti-rabbit IgG, diluted 1/200 (Vector) and preabsorbed with guinea pig serum were used to stain bone marrow. The peroxidase reaction was developed with diaminobenzidine and imidazole, and counterstained with hematoxylin and briefly with eosin. Arrowheads indicate megakaryocytes. Similar bone marrow preparations in rats indicated fibrinogen localization in rat megakaryocytes.

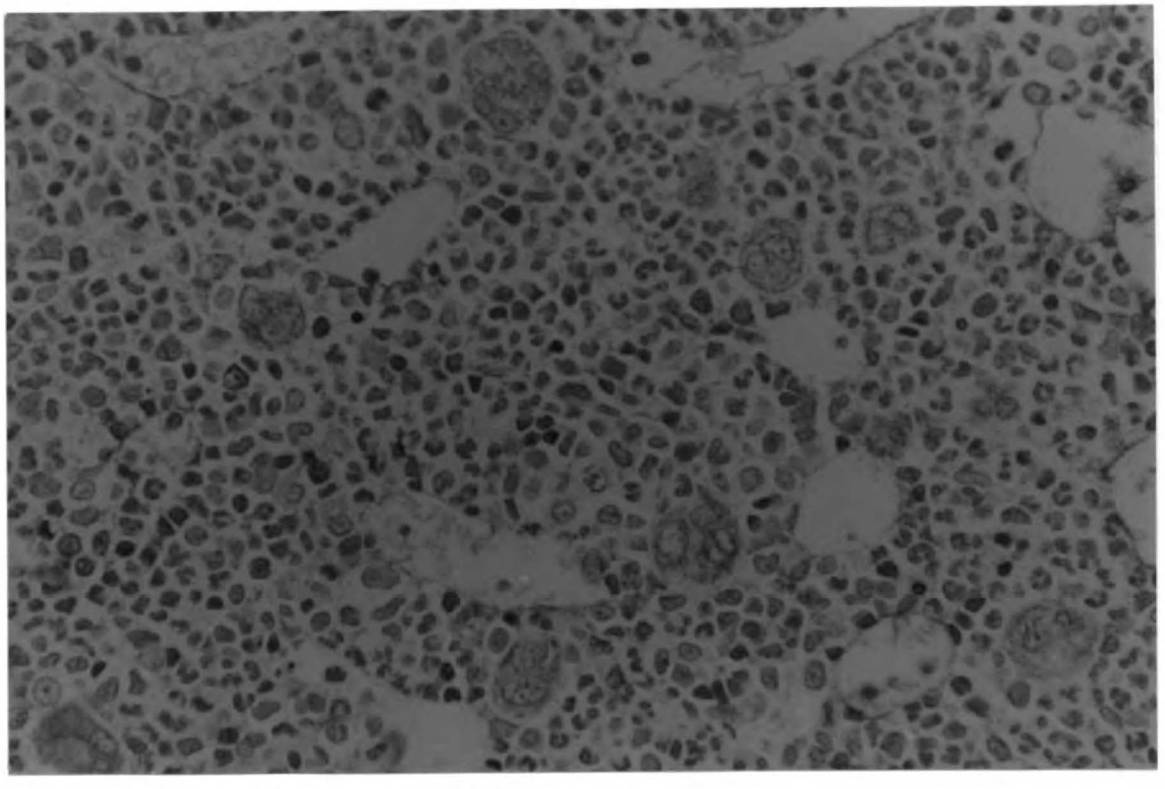


Figure 4

Megakaryocytes were isolated from rat bone marrow by Percoll density gradient fractionation, and determined to be approximately 90% pure by morphologic criteria (Handagama et al., 1987). RNA was isolated, from 1,000-10,000 cells, reverse transcribed, and amplified by PCR as described in Chapter 2. The structures of amplified structures and oligonucleotide primers are given in Figure 1. α-fibrinogen transcripts were not detected in 1,000 16-ploid megakaryocytes although a similarly primed PCR reaction detected this mRNA in 100 diploid hepatocytes (Figs. 2,3). In a separate experiment 10,000 mekaryocytes produced no α fibrinogen signal in the PCR (data not shown). In contrast, α-fibrinogen protein was detected in rat bone marrow megakaryocytes (Fig. 4). Although the protein is present we concluded that there was either no transcript present, or that it was less than about 100-fold abundant per cell than in hepatocytes and thus unlikely to be sufficiently abundant to account for platelet fibrinogen. However, no estimate of number of fibrinogen mRNA copies/hepatocyte is available and an estimate for the minimum fibrinogen mRNA/megakaryocyte is not possible. Further evidence supporting an exogenous source for platelet fibrinogen include observation of uptake of labeled fibrinogen into megakaryocytes in vivo, and depletion of megakaryocyte fibrinogen by decreasing plasma fibrinogen with snake venom (Handagama, personal communication). Decreasing plasma fibrinogen by snake venom treatment did not decrease hepatocyte synthesis of fibrinogen.

Taken together these data suggest that the fibrinogen protein found in megakaryocytes is synthesized elsewhere and taken up by megakaryocyte. Exogenous albumin is taken up by megakaryocytes, and ultimately is found in platelets (Handagama *et al.*, in press). Like α-fibrinogen, albumin is synthesized in liver and large quantities are secreted into blood. The transcript for albumin was not detectable in 2,000 megakaryocytes, but was found in liver (Figs. 7,8). The sensitivity of albumin transcript detection in liver is 1 pg whole cellular RNA (equivalent to 1/10 of a single hepatocyte). We think it is unlikely that albumin is transcribed

Figure 5. Rat megakaryocytes contain mRNA transcripts for β-actin and PDGF-A. RT-PCR products derived from the RNA fron 1,000 megakaryocytes/lane were electrophoresed on 4% agarose and ethidium bromide stained. M=1 kb ladder (marker sizes are given in Chapter 2, figure 4) lanes 1-4=β-actin, lanes 5-8=PDGF A. Each lane represents RNA purified separately from a single cellular prep. This experiment has been repeated 3 times. Bands were validated by size and by restriction analysis.

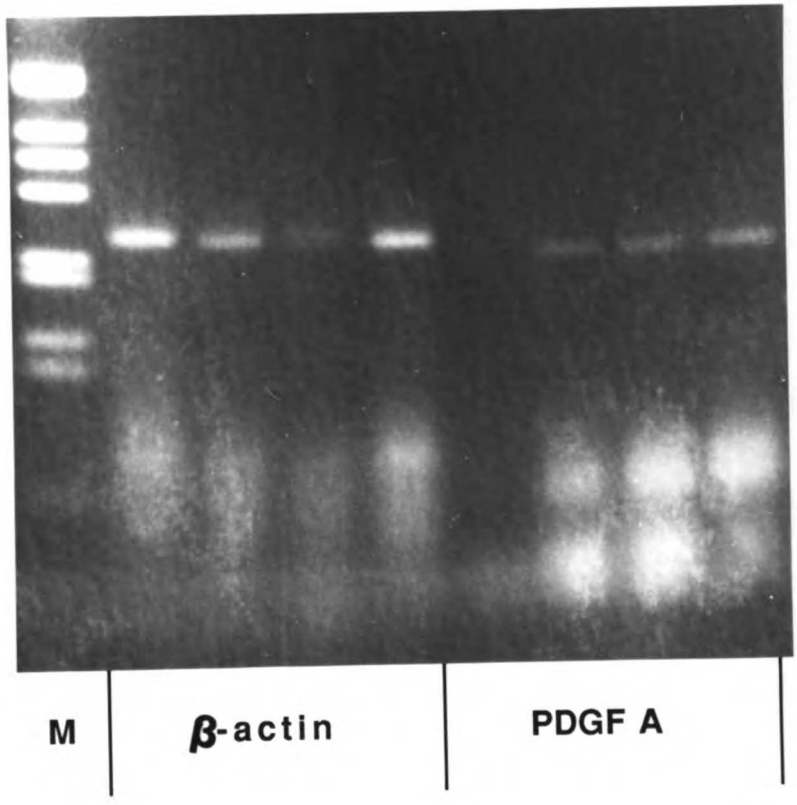


Figure 5

Figure 6. Rat megakaryocytes do not express EGF mRNA. RT-PCR products derived from 1,000 megakaryocytes or 1 ng submaxillary gland were electrophoresed on 4% agarose and ethidium bromide stained. M=1 kb ladder, lanes 1-4=megakaryocytes, lanes 5-7=submaxillary gland.

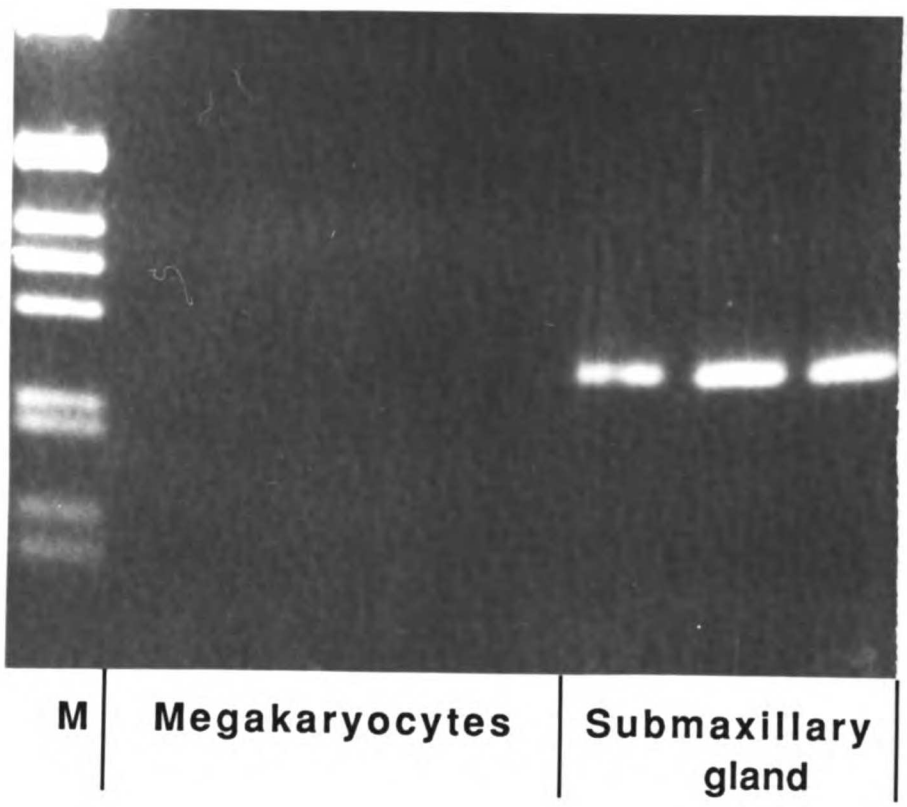
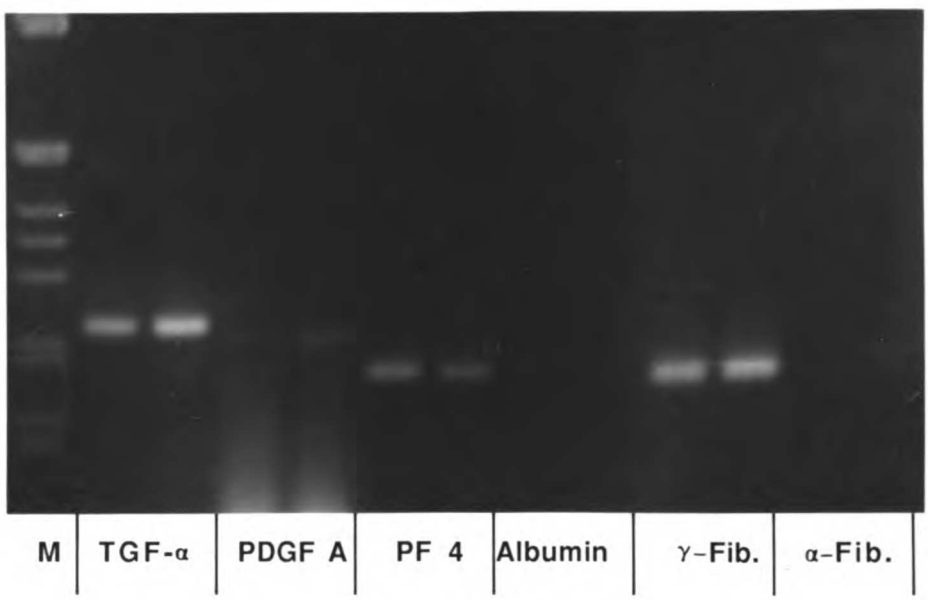


Figure 6

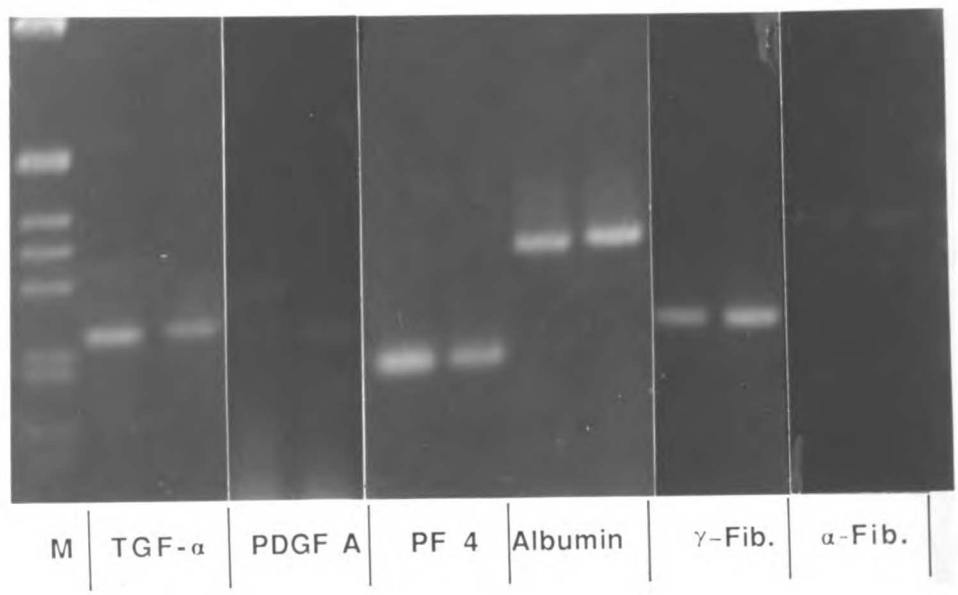
Figure 7. Rat megakaryocytes express mRNA transcripts for TGF-α, PDGF A, PF4, and an anomalously small γ-fibrinogen, but not albumin and α-fibrinogen. In 3 other experiments γ-fibrinogen were the predictede size, that of liver γ-fibrinogen fragments (see figure 8). RT-PCR products derived from 2,000 megakaryocytes/lane were electrophoresed on 4% agarose and ethidium bromideestained (60 cycles of PCR). Each lane represents RNA purified separately from a single cellular prep. This experiment has been repeated 2 times from separate cellular preps. Albumin positive control is in figure 8.



Megakaryocyte (RNA from 2000 cells)

Figure 7

Figure 8. Liver contains mRNA transcripts for albumin, α-fibrinogen, and γ-fibrinogen. RT-PCR products from RNA from 20,000 liver cell equivalents was electrophoresed on 4% agarose and ethidium bromide stained. All preparations done in duplicate.



Liver **(RNA from 20000 cells)**

Figure 8

by rat megakaryocytes, although albumin protein is localized in these cells (Fig. 9). One surprising observation was that of PF 4 mRNA was found in liver cells. The expression of PF 4 observed in liver could be due to the occasional megakaryocytes in liver, or contaminating platelets with residual mRNA. However, bile canaliculi in liver are positive for PF 4 by immunocytochemistry (D. Bainton, personal communication). Megakaryocytes that were negative for α-fibrinogen and albumin transcripts contained β-actin, PDGF-A, and TGF-α, but not EGF transcripts (Figs. 2,5,6). These data correlate with protein phenotype expressed in platelets (Stiles, 1983; Rik Derynck, personal communication). Our observations provide evidence that TGF-α, not EGF is transcribed by megakaryocytes. It is interesting to note the similarity in the growth factor mRNA transcript phenotypes of macrophages and platelets. Both cell types participate in hemostatic, inflammatory, and wound healing functions.

Our results indicate that megakaryocyte α granules contain 2 proteins, albumin and α-fibrinogen, which are derived from exogenous sources. Each of these proteins is synthesized in liver, and is present at high concentration in blood (approximately 40 mg/ml and 1 mg/ml, respectively). Other α granule proteins, such as PDGF, are transcribed, and probably translated in megakaryocytes, and are found at low concentrations in blood (1 ng/ml, or 10^{-7} of the concentration of plasma fibrinogen).

The mechanism of endocytosis of albumin or fibrinogen into α granules is not understood. Specific receptors for fibrinogen (GP Ib-1X, GP IIb-IIIa) exist in megakaryocytes (Levesque, 1987), but it is unknown if they function in the receptor-mediated endocytosis of plasma fibrinogen. Specific receptors for albumin have not been reported in megakaryocytes.

We are currently addressing contradictions between our results and previously published reports. Fibrinogen transcripts and biosynthetically labelled fibrinogen have previously been detected in impure bone marrow derived megakaryocyte preparations (Uzan et al., 1986,

Figure 9. Guinea pig megakaryocytes contain albumin protein. Whole bone marrow was prepared as described by Beckstead et al. (1986). Rabbit anti-albumin diluted (Cappel) and HRP-conjugated anti-rabbit IgG diluted 1/200 (Vector) preabsorbed with guinea pig serum, were used to stain the bone marrow cells. The peroxidase reaction was developed with diaminobenzidine and imidazole, and counterstained with hematoxylin, and briefly with eosin. Arrowheads indicate megakaryocytes. Similar bone marrow preparations from rats indicated localization of albumin in megakaryocytes.

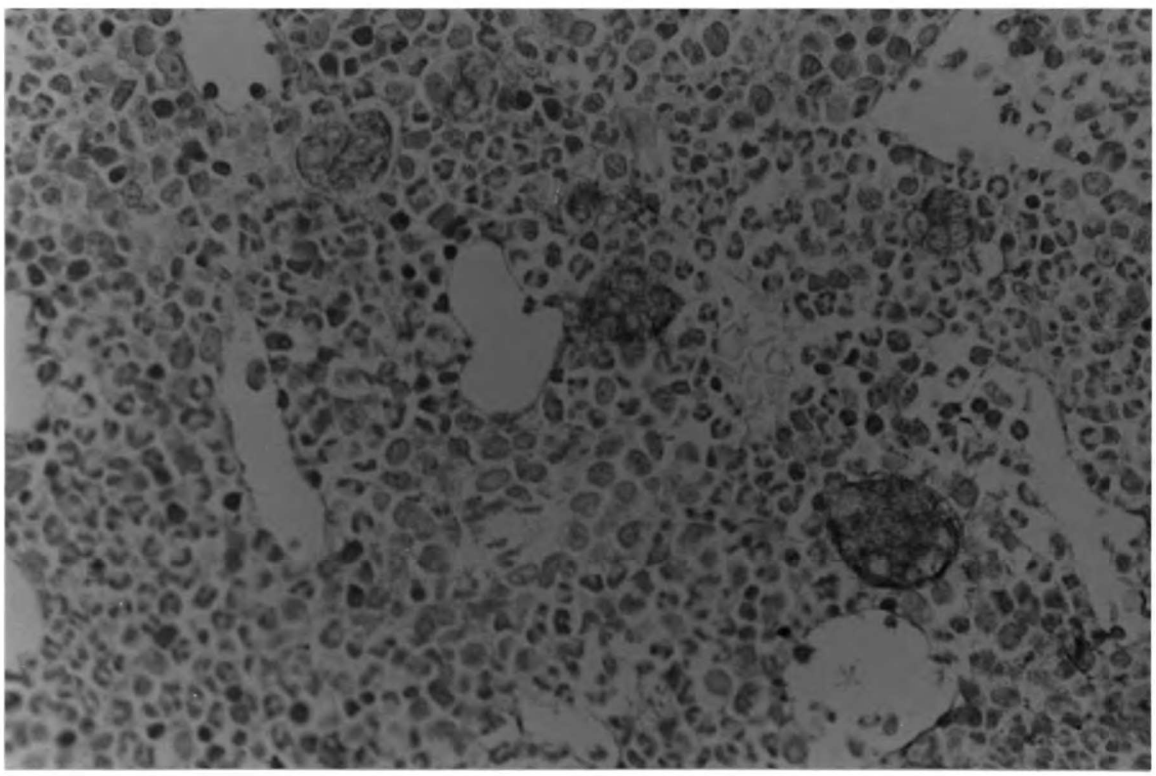


Figure 9

Leven et al., 1985, Belloc et al., 1985). One possibility is that fibrinogen synthesis by bone marrow cells (megakaryocytes or other cells) is regulated by substances such as LPS, and thus differences in the health status of animals in our study and previous studies could give rise to the different results. We plan to test this by injecting LPS into rats 24 hr prior to isolation of megakaryocytes and megakaryocyte-depleted bone marrow cells. We plan to ascertain whether other cells in rat bone marrow transcribe fibrinogen. Preliminary evidence suggests that megakaryocyte cell lines do not transcribe either α or γ fibrinogen. In mice, elicited peritoneal macrophages transcribe γ, but not α fibrinogen (data not shown). Macrophages have not previously been reported to synthesize fibrinogen. Therefore, it is possible that there is a contaminating cell type in bone marrow which produces α- and γ- fibrinogen. Two questions arise; do LPS stimulated macrophages transcribe α and γ fibrinogen mRNA and do they translate these into proteins? To address these questions rat bone marrow macrophages and elicited peritoneal macrophages will be tested for α and γ fibrinogen mRNA transcripts and biosynthetically labelled α and γ fibrinogen protein.

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Appendix C

Secretory Products of Phagocytes

Introduction

The professional phagocytes of the body are equipped to maintain homeostasis upon challenge by foreign organisms or trauma. There are two types of professional phagocytes, polymorphonuclear leukocytes (PMN) and macrophages. PMN are short-lived cells, present at high concentration, with a limited number of prepackaged secretory products that mediate acute inflammation and killing. Macrophages are fewer in number and longer-lived, with a peak response later after challenge than PMN; they participate in both acute and chronic inflammation. Macrophages are ubiquitous in the body as resident fixed tissue macrophages, such as Kupffer cells, Langerhans cells, histocytes and microglial cells, serosal cavity macrophages, and macrophages elicited by inflammation, and have a variety of constitutive as well as inflammation-induced phenotypes. Both types of phagocytes are produced from stem cells in the bone marrow. Reflective of their respective functions, PMN are stored in large reserves in bone marrow and their release is induced by the earliest inflammatory signals, whereas new pools of monocytes are generated by inflammatory signals but are not held in bone marrow reserves under normal conditions. Responses to challenge occur in five temporal segments: inflammation, killing, immunity, debridement, and wound healing. PMN mediate the first two responses and macrophages mediate all five responses.

The secretory products of each cell type (Tables 1 and 2) reflect the responses that each phagocyte mediates. Recent research into the PMN products has uncovered a family of small antimicrobial polypeptides called defensins, has identified the locus and product of the X-linked chronic granulomatous disease (X-CGD), which has a respiratory burst dysfunction, and has elucidated the structure and function of several enzymes found in PMN granules. Recent research into the macrophage secretory products has uncovered an array of pleiotrophic polypeptide growth factors: TGF-α, TGF-β, CSF-1, GM-CSF, IL-6/HSF/BSF-2/IFN-β_2, IGF-I, bombesin, and G-CSF. A diagram illustrating the complex interactions of

Table 1. Secretory products of macrophages.

Polypeptide hormones

Interleukin 1-α and 1-β (collectively, IL-1)

*Somatotropin

Tumor necrosis factor-α (cachectin) (TNF-α)

Interferon-α (IFN-α)

*Interleukin 6/hepatocyte stimulating factor/β-cell stimulating factor-2/interferon-β_2 (IL-6/HSF/BSF-2/IFN-β_2)/plasmacytoma growth factor

Platelet-derived growth factor(s) (PDGF)

Substance P

Fibroblast growth factors (FGF)

Fibroblast activating factors

Transforming growth factor-β (TGF-β)

*Insulinlike growth factor I (IGF-I)

Thymosin B4

*Erythropoietin (EPO)

*Colony-stimulating factor for granulocytes and macrophages (GM-CSF)

*Colony-stimulating factor for granulocytes (G-CSF)

*Colony-stimulating factor for macrophages (M-CSF, CSF-1)

Bombesin

Erythroid colony-potentiating factor (EPA)/tissue

inhibitor of metalloproteinases (TIMP)

Factor-inducing monocytopoiesis (FIM)

β-Endorphin

Adrenocorticotrophic hormone

Neutrophil-activating factor

*Transforming growth factor-α (TGF-α)

Complement (C) components

*Classical pathway: C1, C4, C2, C3, C5, C6, C7, C8, C9;

active complement fragments generated by macrophage proteinases:

C3a, C3b, C5a, Bb

Alternative pathway: factor B, factor D, properidin

Inhibitors: factor I (C3b inactivator), factor H (β-1H)

Coagulation factors

Intrinsic pathway: IX, X, V, prothrombin

Extrinsic pathway: VII

Surface activities: tissue factor, prothrombinase

Antithrombolytic activities: plasminogen activator inhibitor-2,

plasmin inhibitors

Proteolytic enzymes

*Metalloproteinases: macrophage elastase, collagenase, stromelysin,

92 kDa gelatinase, 68 kDa gelatinase, angiotensin convertase

Serine proteinases: urokinase-type plasminogen activator (uPA),

cytolytic proteinase

Aspartyl proteinases: cathepsin D

Cysteine proteinase: cathepsin L, cathepsin B

Other enzymes

Lipases: lipoprotein lipase, phospholipase

Glucosaminidase: lysozyme

Lysosomal acid hydrolases: proteases, lipases, (deoxy)ribonucleases, phosphatases, glycosidases, sulfatases (approximately 40)

Deaminase: arginase

Inhibitors of enzymes

Proteinase inhibitors: α_2-macroglobulin, α_1-proteinase inhibitor (α_1-PI)/α_1-antitrypsin, plasminogen activator inhibitor-2 (PAI-2), plasmin inhibitors, tissue inhibitor of metalloproteinases (TIMP)/ collagenase inhibitor/EPA

Phospholipase inhibitor: lipomodulin (macrocortin)

Proteins of extracellular matrix or cell adhesion

Fibronectin

Gelatin-binding protein/92 kDa gelatinase

Thrombospondin

Chondroitin sulfate proteoglycans

Heparin sulfate proteoglycans

Other binding proteins

For metals: transferrin, acidic isoferritins

For vitamins: transcobalamin II

For lipids: apolipoprotein E, lipid transfer protein

For growth factors: α_2-macroglobulin, IL-1 inhibitors,

TGF-β-binding protein

For biotin: avidin

Bioactive lipids

Cyclooxygenase products: prostaglandin E_2 (PGE_2),

prostaglandin $F_{2\alpha}$, prostacyclin (PGI_2), thromboxane

Lipoxygenase products: monohydroxyeicosatetraenoic acids (HETE),

dihydroxyeicosatetraenoic acids, leukotrienes (LT) B4, C, D, E

Platelet-activating factors (PAF)

(1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine)

Other bioactive low molecular weight substances

Oligopeptides: glutathione

Steroid hormones: 1α, 25-dihydroxyvitamin D3

Purine and pyrimidine products: thymidine, uracil, uric acid, deoxycytidine, cAMP,

neopterin (2-amino-4-oxo-6-trihydroxypropylpteridine)

Reactive oxygen intermediates: superoxide, hydrogen peroxide, hydroxyl radical,

singlet oxygen, hypohalous acids

Reactive nitrogen intermediates: nitrites, nitrates

This table was adapted from Nathan (3), and additional information was obtained from Takemura and Werb (*Am J Physiol* 1984, 246:C1-C9), Rappolee and Werb (unpublished observations), and the annotated references. Some of the products listed require confirmation as genuine biosynthetic products. The asterisks indicate newly described products.

Table 2. Secreted and granule-associated products of PMN.

PMN elastase
Cathepsin G
Myeloperoxidase
Lysozyme
Lysosomal acid hydrolases
92 kDa gelatinase/gelatin binding protein
PMN collagenase
Lactoferrin
Lysozyme
Defensins
Plasminogen activator
Reactive oxygen metabolites ($O^{\cdot}_2$, H_2O_2, HOCl, $^{\cdot}OH$)
Active lipid metabolites
Fibronectin

This is a restricted list of PMN products that have been observed extracellularly.

growth factors with target cells is shown in Fig. 1. In addition, recombinant TNF-α and IL-1, mice transgenic for GM-CSF, and tumor cells transfected with TNF-α have been used to study the activities of these macrophage secretion products in a manner more precise than previously possible.

Inflammation

PMN and macrophages are attracted by and respond to inflammatory reactants such as leukotrienes and complement split products. PMN respond in minutes to hours and monocytes-macrophages in hours to days. The PMN response is relatively simple: inflammatory signals induce adhesiveness, chemoattraction, activation of the microbicidal respiratory burst, and degranulation (Fig. 2), which releases hydrolytic enzymes and antimicrobial polypeptides. The response of monocytes and macrophages to inflammatory stimuli is slower and more complex. Many inflammatory factors are produced by macrophages (Table 1). Recently, the paracrine sources of these factors and their effects on target cells have been studied (Fig. 1). TNF-α and IL-1 induce adhesiveness, fever, the acute-phase response in liver, induction of the immune response, and stimulation of secretion (1-5). Although macrophages are the primary source of IL-1, TNF-α induces IL-1 on fibroblast and endothelial cell membranes (6). In addition, monocyte chemoattractants, such as platelet TGF-β (7) and C5a (8), induce macrophage IL-1. Immune IFN-γ is synergistic with C5a in induction of macrophage IL-1. At inflammatory foci, macrophages attract more macrophages by production of TGF-β (9). On the molecular level an example of positive feedback is the induction of IL-1 by macrophage IL-1 (10).

A number of hematopoietic growth factors, called colony-stimulating factors, activate phagocytes and stimulate their production in bone marrow. IL-1 and TNF-α have recently been shown to stimulate accumulation of mRNA for GM-CSF, G-CSF, and M-CSF in endothelial cells (11). M-CSF is a growth and activation factor specific for cells of the

Figure 1. A diagram of the bidirectional interactions of growth control factors secreted by macrophages on target cells.

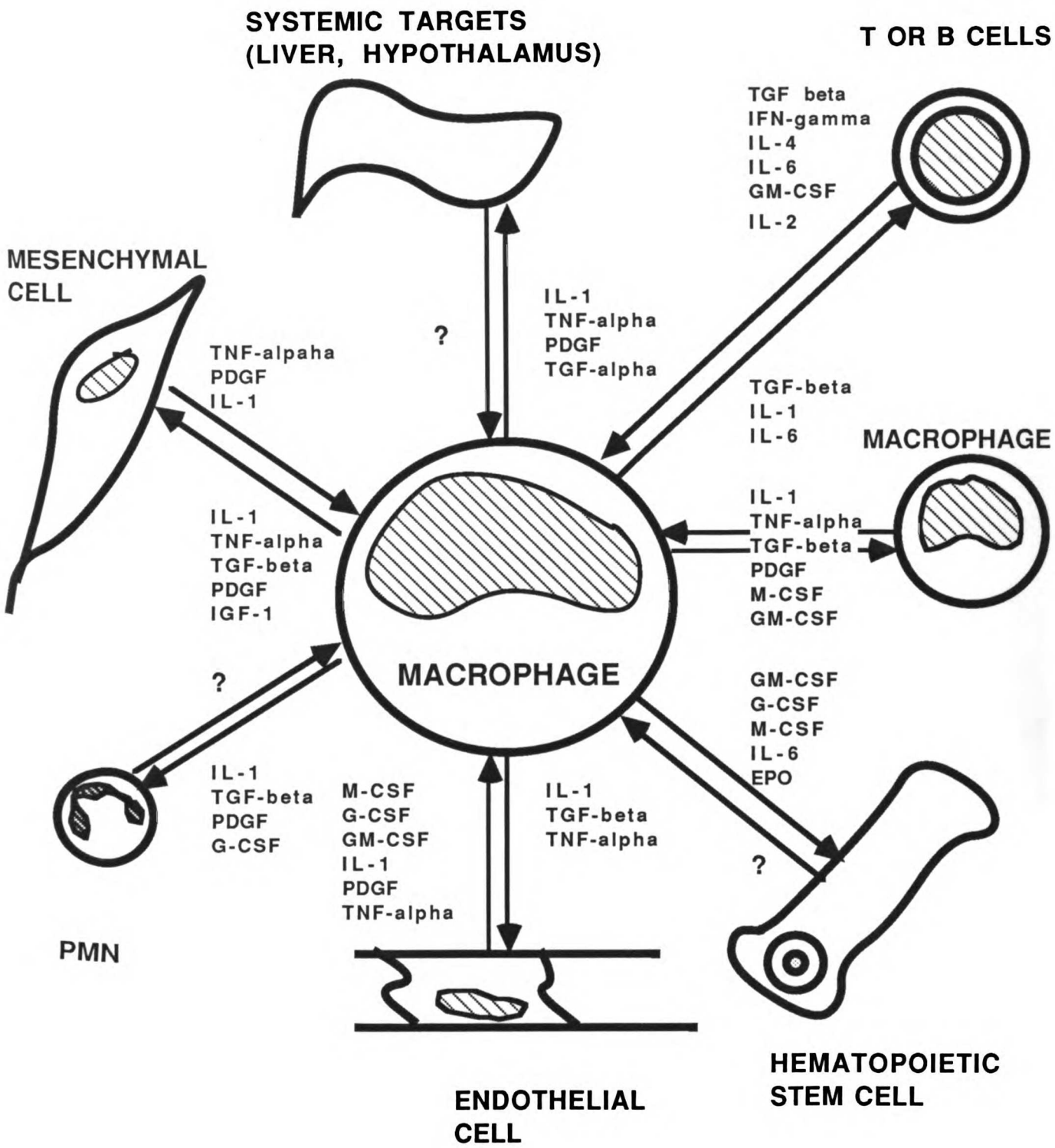

SYSTEMIC TARGETS
(LIVER, HYPOTHALAMUS)
T OR B CELLS
TGF beta
IFN-gamma
IL-4
IL-6
GM-CSF
IL-2
MESENCHYMAL
CELL
?
IL-1
TNF-alpha
PDGF
TGF-alpha
TNF-alpaha
PDGF
IL-1
TGF-beta
IL-1
IL-6
MACROPHAGE
IL-1
TNF-alpha
TGF-beta
PDGF
M-CSF
GM-CSF
IL-1
TNF-alpha
TGF-beta
PDGF
IGF-1
MACROPHAGE
?
GM-CSF
G-CSF
M-CSF
IL-6
EPO
IL-1
TGF-beta
PDGF
G-CSF
M-CSF
G-CSF
GM-CSF
IL-1
PDGF
TNF-alpha
IL-1
TGF-beta
TNF-alpha
?
PMN
ENDOTHELIAL
CELL
HEMATOPOIETIC
STEM CELL

Figure 2. A scanning electron micrograph of PMN stimulated to secrete their granular contents by treatment with 1 nM formyl-methionyl-leucyl-phenylalanine.

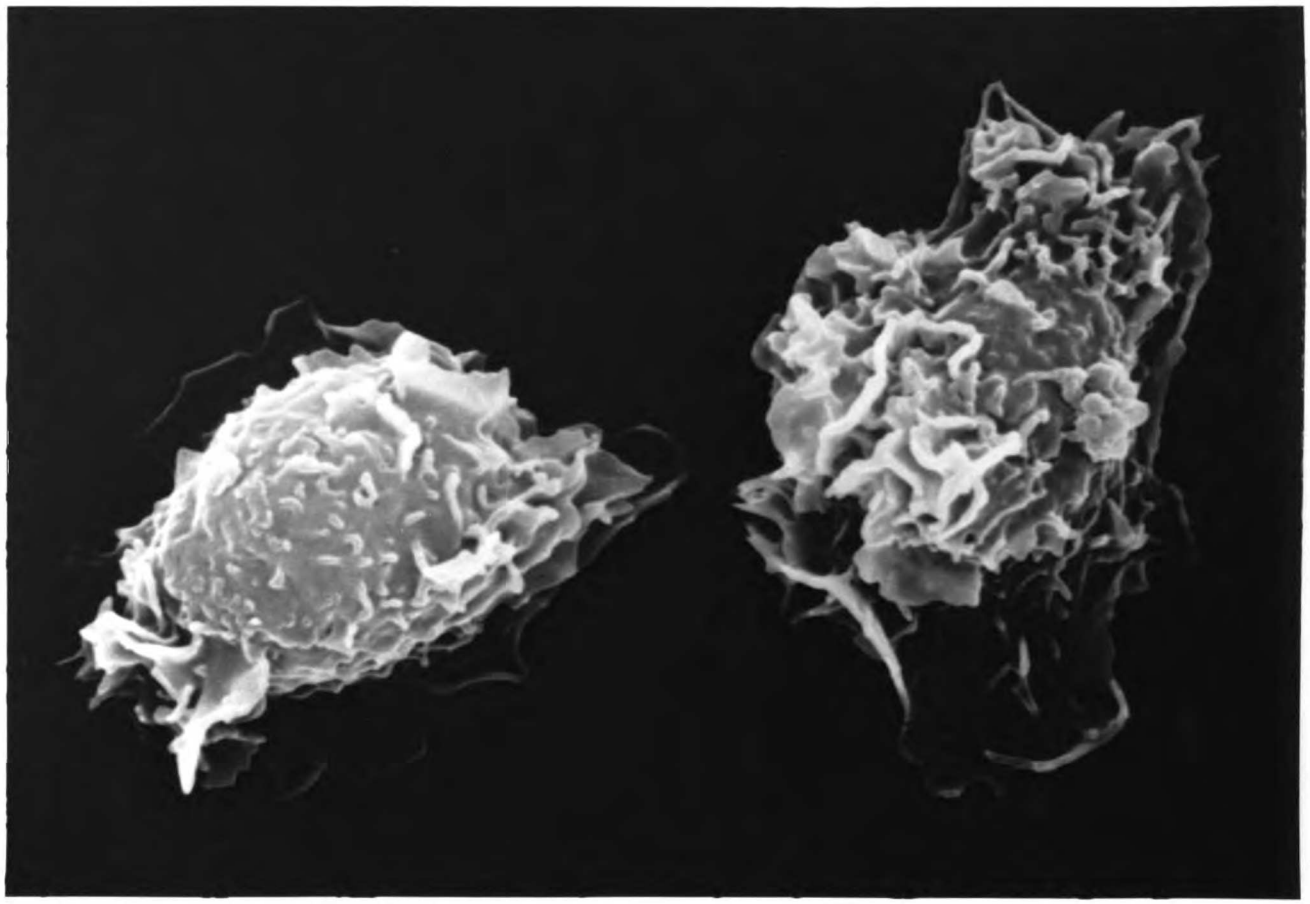


Figure 2

monocyte lineage. The plasma concentration of this factor rises during inflammation but may be controlled by macrophage number (12). IFN-γ and GM-CSF induce macrophages themselves to express M-CSF mRNA and activity (13,14), suggesting an autocrine stimulation via this factor. Several inflammatory stimuli cause macrophages to express GM-CSF mRNA and activity (15). That GM-CSF can induce further macrophage production is demonstrated in transgenic GM-CSF mice, in which high GM-CSF production results in elevated plasma GM-CSF and multiple foci of macrophage accumulations (16). GM-CSF must also activate macrophages because of the damage seen in tissues accumulating macrophages (16). Similarly, when cells expressing a transfected TNF-α gene were inoculated into nude mice, TNF-α caused wasting and death (17).

Killing

Phagocytes kill by means of plugs (complement), burns (reactive oxygen intermediates), or poisons (TNF-α, cytolytic proteinases) (Lachmann, *Nature* 1986, 321: 560). As soon as phagocytes reach a nonsterile inflammatory focus, a two-step process leads to killing of microorganisms by oxygen metabolites (Adams and Hamilton, *Annu Rev Immunol* 1984, 2: 283-318). In the case of X-CGD, PMN and monocytes-macrophages ingest, but do not kill, certain bacteria because they are unable to mount a respiratory burst (18). The gene defect in this disease involves cytochrome b_{-245}, which is a link in the biochemical pathway of the oxidative burst. The gene defect is not in the cytochrome b_{-245} subunit itself but in an accessory subunit (19,20). The cloning of this gene promises a gene therapy approach to curing this disease.

PMN produce a group of cytostatic and cytocidal polypeptides such as lactoferrin and lysosomal hydrolases. Recently, a class of small microbicidal polypeptides called defensins was discovered (Ganz *et al., J Clin Invest* 1985, 76:1427-1435) and found to be stored in a subclass of PMN azurophil granules (21).

Lymphokines and monokines are involved in the induction of microbicidal killing and related tumoricidal killing (1,22,23). TNF-α and IFN-γ are synergistic in their induction of tumoricidal activity in macrophages (24). This indicates that inflammatory and immune signals combine to induce tumoricidal activity via different intracellular pathways. A third, endocrine pathway for priming for tumoricidal activity is indicated by the recent studies showing that somatotropin is as effective as IFN-γ in priming macrophages for superoxide production (25). It is possible that somatotropin is also a product of leukocytes. Recent work has also shown that macrophages produce complement components C6, C7, C8, and C9, suggesting that macrophages may be capable of generating a complete complement cascade resulting in cytolysis (26). Since macrophages also synthesize C3, C4 and C5, they produce all factors of the alternate pathway of the complement cascade. This cascade is activated by bacterial and yeast cell walls.

Immunity

Macrophages are important accessory cells in the generation of the humoral immune response. Not only do macrophages present digested antigenic epitopes complexed with Ia antigens that can be induced by IFN-γ, but the cytokines produced by macrophages facilitate the progression of the immune response. IL-1 enhanced immune reactions in both T and B cells (27). Activated T cells produce GM-CSF, which induces macrophages to produce autostimulatory M-CSF (13). In addition, the B-cell stimulating factor-2 (BSF-2) produced by macrophages was recently shown to be identical to macrophage IFN-β_2, HSF-2, and IL-6 (28,29). IFN-β_2 is also a competence gene induced by PDGF and is thought to mediate negative feedback to PDGF growth induction.

Debridement

Both macrophages and PMN participate in remodeling of sites of injury by their

secretion of proteolytic enzymes that can be active in tissue spaces and by endocytosis and intracellular degradation of the extracellular matrix and cellular debris.

In PMN the metalloproteinase-degrading enzymes PMN collagenase (30) and 92 kDa gelatinase (Hibbs *et al., J Biol Chem* 1985, 260:2493-2500) are found in specific granules, and cathepsin G and PMN elastase are found in azurophil granules. Recent experiments have determined the primary structure of cathepsin G (31) and PMN elastase (32). With this information therapeutic inhibitors for these enzymes can be designed.

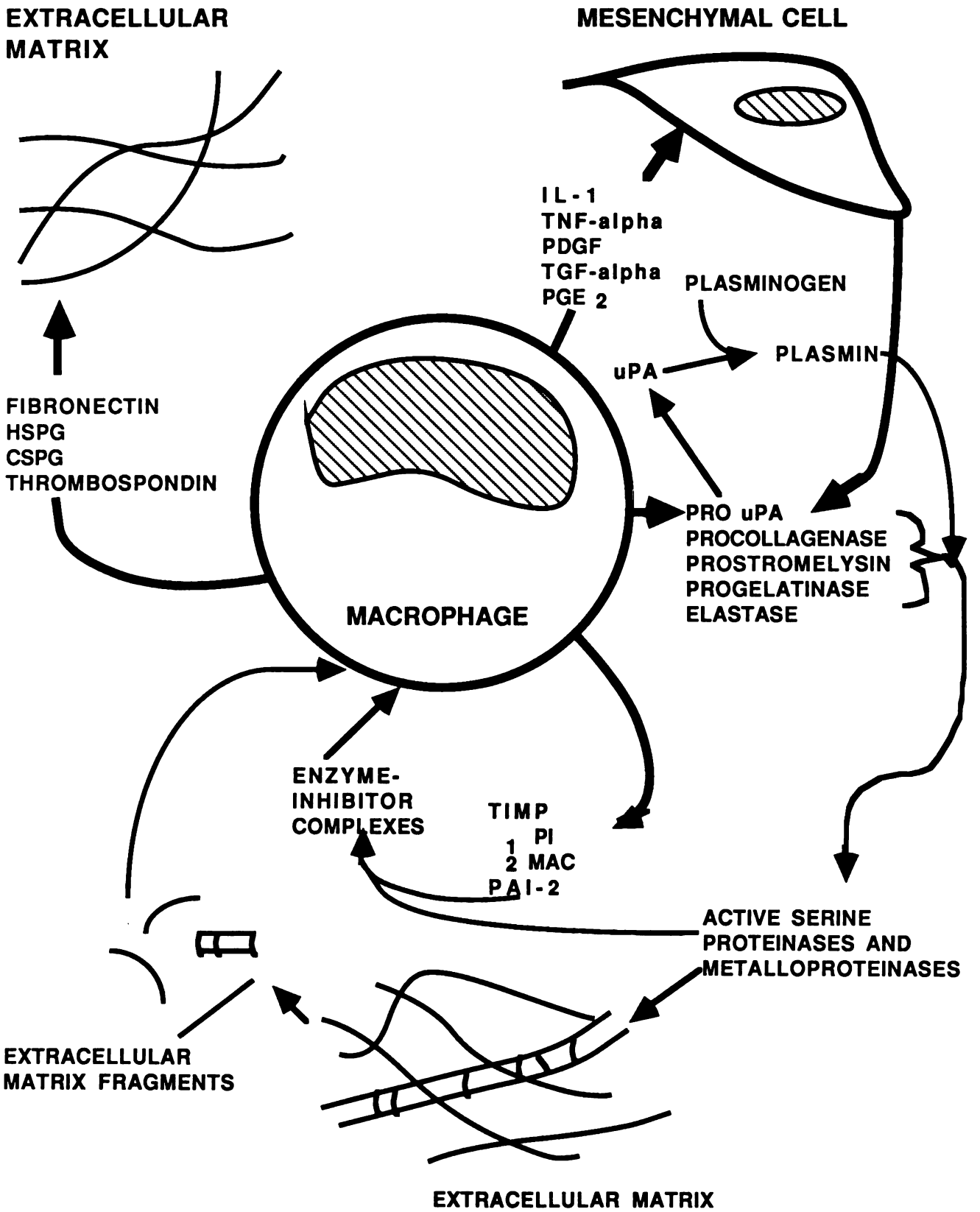
Monocytes contain cathepsin G and PMN elastase in their azurophil granules (Welgus *et al., J Clin Invest* 1986, 77:1675-1681). Once they become macrophages they can be stimulated by endotoxin to produce the metalloproteinases collagenase (33) and stromelysin (34) and their inhibitor TIMP (33). In addition, stimulated macrophages secrete urokinase and have a membrane receptor that focalizes this proteinase to the macrophage surface (35).

In addition to secreting extracellular matrix-degrading enzymes, macrophages also regulate the expression of these enzymes by fibroblasts and other cells through their secretion of inducing factors, including IL-1, TNF-α, and PDGF (Murphy *et al., J Biol Chem* 1985, 260:3079-3083; Dayer *et al., J Exp Med* 1985, 162:2163-2168; Bauer *et al., Proc Natl Acad Sci USA* 1985, 82:4132-4136). The pathway for macrophage secretion products in debridement is shown in Fig. 3.

Wound Healing

Leibovich and Ross (*Am J Pathol* 1975, 78:71-100) showed that macrophages are required for proper wound healing. Recently, several wound healing factors have been found in stimulated macrophages. TGF-α mRNA and polypeptide is found in stimulated macrophages and conditioned medium (36,37) and in macrophages isolated from wounds (37). TGF-α is known to be angiogenic (38) and to stimulate epidermal regrowth and

Figure 3. Pathways for extracellular matrix remodeling regulated by macrophage secretion products. Bold arrows indicate secretion, light arrows indicate effector pathways.


EXTRACELLULAR MATRIX
MESENCHYMAL CELL
IL-1
TNF-alpha
PDGF
TGF-alpha
PGE 2
PLASMINOGEN
PLASMIN
uPA
FIBRONECTIN
HSPG
CSPG
THROMBOSPONDIN
PRO uPA
PROCOLLAGENASE
PROSTROMELYSIN
PROGELATINASE
ELASTASE
MACROPHAGE
ENZYME-INHIBITOR COMPLEXES
TIMP
PI
1
2 MAC
PAI-2
ACTIVE SERINE PROTEINASES AND METALLOPROTEINASES
EXTRACELLULAR MATRIX FRAGMENTS
EXTRACELLULAR MATRIX

formation of granulation tissue (Schultz, *Science* 1987, 235:350-352; Buckley *et al.*, *Proc Natl Acad Sci USA* 1985, 82:7340-7344). In addition, activated macrophages synthesize TGF-β (9), which is a chemoattractant for macrophages (7) and is induced in many phases of wound healing (39). Angiogenesis induced by TNF-α has been studied by two groups (40,41), with slightly different interpretations. Leibovich *et al.* (41) conclude that TNF-α is not mitogenic for vascular endothelial cells but can induce limited noninflammatory angiogenesis through chemotactic extension of extant endothelial cells. However, the more likely interpretation comes from the study of Frater-Schröder *et al.* (40), which shows that TNF-α blocks both baseline and FGF-induced endothelial cell growth and that angiogenesis may be induced through the indirect inflammatory effects of TNF-α. Macrophages also produce other factors that may participate in wound healing, including PDGF (Shimokado *et al.*, *Cell* 1985, 43:277-286; Martinet *et al.*, Nature 1986, 319:158-160) and bombesin (Wiedermann *et al.*, *J Immunol* 1986, 137:3928-3932).

In a form of wound healing involving macrophage debridement of crushed nerve, the peak induction of NGF mRNA is correlated with macrophage infiltration and debridement of injured nerve sheaths. The macrophages become loaded with lipid debris from the myelin sheaths and are stimulated to produce very large amounts of apolipoprotein E, which becomes the predominant newly synthesized protein in the sites of injury (Ignatius *et al.*, *Science* 1987, 236:959-962). Macrophages also express the LDL receptor, which can take up its own apolipoprotein E, suggesting another type of autocrine loop. Lipid loading of macrophages by uptake of lipoproteins also induces the production of TGF-α and IL-1 (37). Macrophages do not produce NGF mRNA, but IL-1 induces a large increase in NGF mRNA in non-neuronal cells of sciatic nerve (42). Similar growth factor cascades initiated by lipid loading of macrophages (37) may contribute to atherogenesis, which may also be initiated by a process akin to wound healing (43).

Another event in the repair process is the synthesis of extracellular matrix components. Macrophages synthesize fibronectin and both chondroitin sulfate and heparin sulfate proteoglycans (44). Because macrophages also produce heparin-binding growth factors, such as FGF and PDGF, these matrix components may regulate growth factor pools.

Summary

Great strides have been made in the understanding of the secretory states and functions of phagocytes. Several bioactivities carried out by macrophage secretory products can be ascribed to molecules recently added to the phagocyte secretory repertoire: TGF-α, TGF-β, GM-CSF, G-CSF, M-CSF, bombesin, and IL-6 The effects and interactions of these factors and of previously described major macrophage secretory products, such as TNF-α and IL-1, have been more precisely determined. The molecular cloning of these factors holds the promise of their clinical application. The elucidation of the X-CGD locus may lead to a gene therapy strategy for this disease. Experimental techniques for single-cell mRNA phenotyping may lead to rapid understanding of the secretory potential of various phagocytes *in vivo* (37).

Much work needs to be done on the singular and integrated effects of phagocyte secretory products. New questions are appearing on the horizon. What is the state of the macrophage amid so many potentially autostimulatory secretion response pathways induced by IL-1 and other monokines? Are PGE_2, lactoferrin, IFN-β_2, and glucocorticoids (45) the main negative regulatory substances, or will others be found? What is the significance of the fact that macrophages become refractory to induction of growth factor transcript accumulation differently with respect to different growth factors? (PDGF-B and GM-CSF mRNA accumulation disappears 1/2 day after stimulation, whereas IL-1α accumulation endures for 24 hours.) What is the significance of post-transcriptional control of the expression of GM-CSF and TGF-β activity? On the cellular level, what controls acute and long-term production of PMN and macrophages? Many macrophage and PMN activities have

not been completely explained on a molecular level. New molecules will be added to the phagocyte secretory repertoire. Those activities with known molecular mediators (e.g., fever is induced by both IL-1 and TNF-α) have not been explained with precision. A numerical understanding of triggering, equilibrium, and decay phases of integrated phagocyte secretory phenotypes will be forthcoming in the near future.

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