ABSTRACT

John Emanuel Poulos. THE EFFECTS OF INDOMETHACIN AND DEXAMETHASONE ON FIBROBLAST CHEMOTAXIS (Under the supervision of Dr. Gerhard Kalmus) Department of Biology, June 1987.

Tissue damage results in an acute inflammatory response followed by a repairative phase which is initiated by fibroblast migration towards chemoattractants released from the wound site. Boyden chambers were employed to investigate the effect of indomethacin and dexamethasone on fibroblast chemotaxis towards conditioned medium. The ability of conditioned medium to serve as a chemoattractant is possibly due to the presence of cell secreted proteins, as well as fibronectin and its fragments. Indomethacin is a non-steroidal anti-inflammatory agent that inhibits prostaglandin synthase whereas, dexamethasone inhibits phospholipase A_2. It was determined that indomethacin did not inhibit but enhanced fibroblast chemotaxis at a concentration of $1x10^{-4}$ to $1x10^{-8}$M. This may be due to the accumulation of arachidonic acid by inhibition of prostaglandin synthase. This arachidonic acid is then free to be acted upon by 5-lipoxygenase thereby producing leukotriene B_4, a potent chemoattractant for fibroblasts. Dexamethasone showed inhibition of fibroblast chemotaxis at $1x10^{-4}$M, and enhancement of chemotaxis at $1x10^{-8}$ to $1x10^{-10}$M. Inhibition may be due to the induction of a chemotactic regulatory protein known as lipocortin. Enhancement may be due to glucocorticoid stimulation of fibronectin synthesis and secretion, accompanied with the upregulation of fibronectin receptors.

THE EFFECTS OF INDOMETHACIN AND DEXAMETHASONE ON FIBROBLAST CHEMOTAXIS

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

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June 1987

The Effects of Indomethacin and
Dexamethasone on Fibroblast Chemotaxis

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ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Gerhard Kalmus for his immense support and supervision during both my undergraduate and graduate career. For it was Dr. Kalmus who was instrumental in encouraging me to pursue a scientific career. I would also like to thank Dr. Jose Caro for giving me the opportunity to work both, on my thesis and as a lab technician in the East Carolina University, Department of Medicine. Extreme thanks also goes out to Dr. Charles O'Rear, who devoted much of his time and effort in reviewing the data presented in this thesis. I wish to also thank Dr. James Smith for providing me with his invaluable assistance and biochemical background.

My extreme gratitude also goes out to Mrs. Karin Kalmus for her expertise on mammalian tissue culture and for providing me with the technical background required for the completion of my thesis. Thanks also goes out to Mr. Jim McLean for his assistance during the final draft of this thesis.

I will always be indebted to the faculty of the Department of Biology for giving me the financial support, facilities, and education required for the attempt and completion of my Master of Science degree in Biology.

Finally, I wish to thank my parents, Mr. and Mrs. Emanuel Poulos who always stood behind me and offered both their love and support in all of my endeavors.

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Abbreviations used in Text

ABP	Actin Binding Protein
aa	amino acids
AA	Arachidonic Acid
BSA	Bovine Serum Albumin
C5a	Complement 5a
CM	Conditioned Medium
FCS	Fetal Calf Serum
Hetes	Hydroxy-eicosatetraenoic acids
LTs	Leukotrienes
MM	Modified Medium
PA_2	Phospholipase A_2
PGs	Prostaglandins
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
SDS	Sodium Dodecyl Sulfate
TxA_2	Thromboxane A_2

INTRODUCTION

Chemotaxis is an important biological phenomenon involving the directed migration of cells along a chemical gradient. This phenomenon is displayed by a wide variety of cells and constitutes a major component of both the inflammatory response and tissue repair. Infection, chemical irritation, and physical injury to tissue and surrounding blood vessels generates localized responses by the host. This localized response involves the release of a wide variety of inflammatory mediators such as: histamine, bradykinin, prostaglandins and leukotrienes. These compounds have the ability to mediate acute inflammation via erythema, edema, platelet aggregation, along with polymorphonuclear and monomorphonuclear cell influx. Following this response, a reparative phase is initiated due to fibroblast recognition of chemoattractants released from damaged tissue and inflammatory cells. These chemoattractants elicit the migration of fibroblasts to the wound site, resulting in cell proliferation, collagen synthesis, and subsequent repair of the connective tissue matrix. Any breakdown in the reparative phase may result in excessive fibroblast chemotaxis and extensive chronic fibrosis. The goal of the present study was to determine if fibroblast chemotaxis could be regulated by inhibition of arachidonic acid metabolism.

HISTORICAL REVIEW

Acute Inflammation

Vasoactive peptides - Any damage to tissue, either due to an immunological or non-immunological stimuli generates a localized response. This response results in activation of the coagulation and complement system. The complement system is only activated upon immunological challenge either from antigen stimulation or bacterial infection. Also occurring is histamine release from mast cells, bradykinin production, arachidonic acid (AA) metabolism, and cell influx. The release of histamine and bradykinin initiates constriction of the vascular endothelial cells, which dilates terminal arterioles and increases the vascular permeability of postcapillary venules. This vasoconstriction followed by vasodilation results in the dilation and loss of fluid through the microvasculature. This is also accompanied by the exudation of plasma proteins in inverse proportion to their molecular size (Magno et al., 1961).

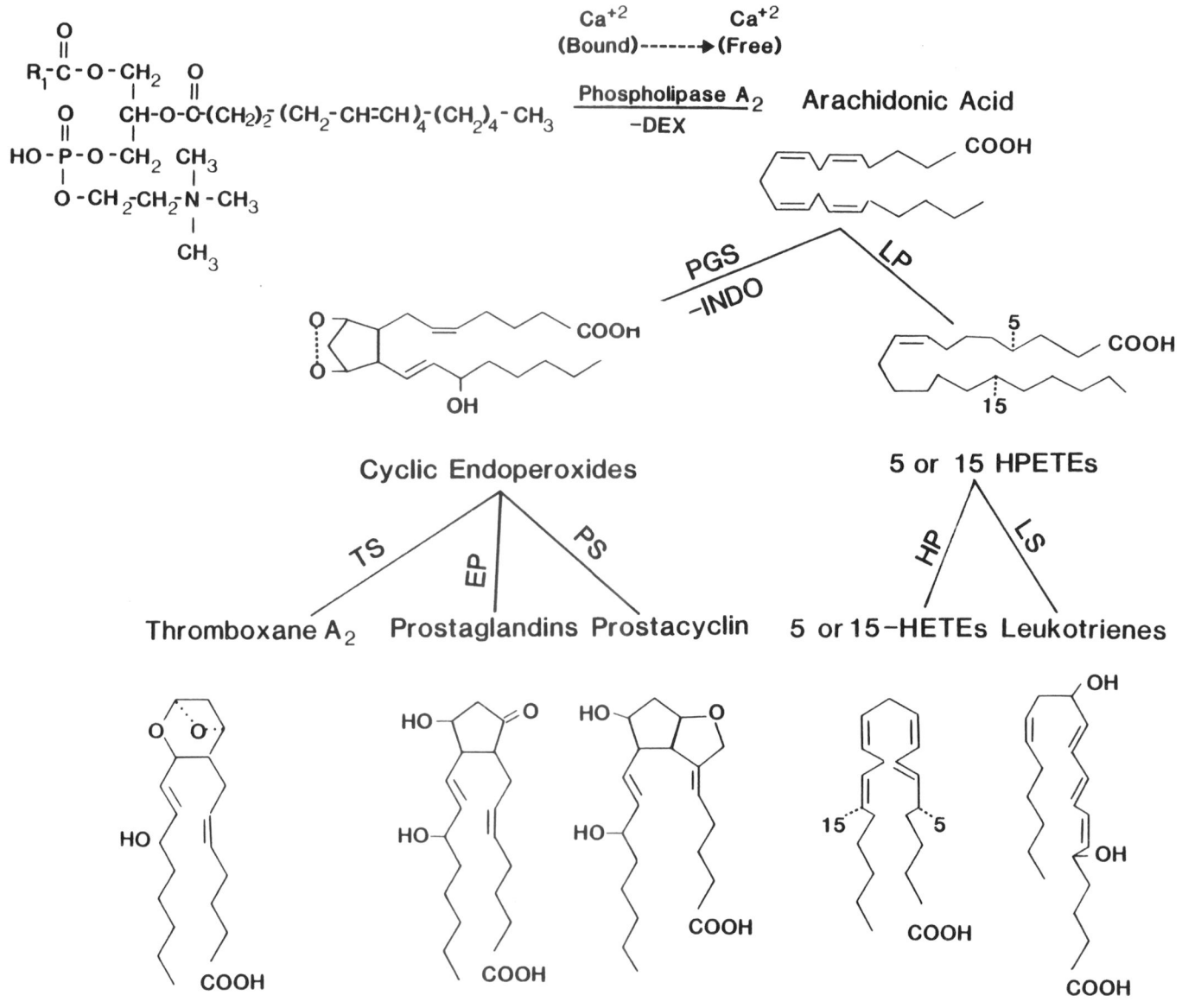
Arachidonic Acid - Also associated with acute inflammation is the enhanced metabolism of AA resulting in the synthesis of prostaglandins (PGs), leukotrienes (LTs), and hydroxy-eicosatetraeonic acids (Hetes). Arachidonic acid is found in abundance in the phospholipids of cell plasma membranes and is esterified at position two of the glycerol backbone. The availability of AA is determined by either the cytidine diphosphate-diacylglycerol pathway or by

the methylation of phosphotidylethanolamine to phosphotidylcholine by methyltransferases. Inhibition of these methylations by agents that raise levels of S-adenosyl homocysteine, block chemotaxis (Schiffman, 1983).

Tissue damage, histamine, Ca^{+2} influx, and bradykinin, all enhance the activity of phospholipase A_2 (PA_2) to release AA and lysophosphotidyl choline. This is the rate limiting step and branch point for the synthesis of PGs, LTs, and Hetes. Removal of the stimulus may result in a rapid reincorporated of AA into the cell membrane. This is accomplished by the action of acyl-CoA synthetase on arachidonic acid to form arachidonyl-CoA. Arachidonyl-CoA is then re-esterified by acyl transferases into the cell membrane. However, in damaged tissues, liberated AA is mainly metabolized in order to mediate inflammation.

Prostaglandins and Thromboxane A_2 - AA may be acted upon by PG synthase to yield PGs, thromboxane A_2 (TxA_2), and prostacyclin (Fig. 1). Prostaglandin synthase is a dimer consisting of two similar 70 Kd subunits and catalyzes both the bis oxygenation of AA to form prostaglandin G_2 (PGG_2) (cyclo-oxygenase) and the peroxide reduction of PGG_2 into prostaglandin H_2 (PGH_2) (Kulmacz, 1985). Cyclo-oxygenase incorporates molecular oxygen into the 20 carbon backbone of AA via a dioxygenase reaction. A hydrogen is abstracted at C-12 and peroxidation occurs at C-11. Molecular O_2 is added at C-15 and ring closure occurs between C-8 and C-12 to form PGG_2. Prostaglandin hydroperoxidase then acts on PGG_2 to

Fig. 1 Arachidonic acid metabolism: Dex, Dexamethasone; PGS, Prostaglandin Synthase; Indo, Indomethacin; HPETEs, Hydroperoxyeicosatetraenoic acid; HP, Hydroperoxidase; LS, Leukotriene Synthetase; HETEs, Hydroxyeicosatetraenoic acid; TS, Thromboxane A_2 Synthetase; EP, Endoperoxide Isomerase; PS, Prostacyclin Synthetase. (-) indicates inhibitor.


Ca+2 (Bound) ------→ Ca+2 (Free)
Phospholipase A2
-DEX
Arachidonic Acid
COOH
PGS
-INDO
LP
COOH
OH
5
COOH
15
Cyclic Endoperoxides
5 or 15 HPETEs
TS
EP
PS
HP
LS
Thromboxane A2
Prostaglandins
Prostacyclin
5 or 15-HETEs
Leukotrienes
HO
COOH
HO
HO
COOH
HO
COOH
15
5
COOH
OH
OH
COOH

form PGH_2. Subsequent enzymatic reactions produce prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$, and TxA_2 (Dawson et al., 1985). Prostacyclin and PGE_2 are potent vasodilators and potentiate the effects of bradykinin and histamine (Higgs et al., 1984). Prostacyclin has the ability to inhibit platelet aggregation and raise c-AMP. Prostaglandin E_2 also has the ability to raise c-AMP and acts as a potent vasodilator (Higgs et al., 1984). Thromboxane A_2 is a potent platelet aggregator and vasoconstrictor, with the ability to mobilize Ca^{+2} from intracellular stores (Gerrard, 1985). This free ionized Ca^{+2} may activate PA_2 or complex with calmodulin to trigger platelet contraction.

Leukotrienes - In neutrophils AA may be oxidized by 12 or 15-lipoxygenase to form Hetes which are potent chemoattractants for neutrophils (Schiffman, 1983). Arachidonic acid may also be acted upon by 5-lipoxygenase to yield leukotriene A_4 (LTA_4). Subsequent enzymatic reactions on LTA_4 produce leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4). The enzymes responsible for the synthesis of the LTs are found in the plasma membrane while the products are found in the cytosol. Leukotriene B_4 is a strong chemoattractant for fibroblasts (Mensing and Czarntzki, 1984) and neutrophils (Ford-Hutchison et al., 1980) and induces muscle contraction and vascular permeability (Gerrard, 1985). Leukotriene B_4 also strongly enhances plasma exudation in combination with bradykinin and enhances Ca^{+2} influx into

cells (Eakins *et al.*, 1980; Molsk *et al.*, 1980); LTC_4 and LTD_4 enhance the adhesiveness of neutrophils (Goetzl *et al.*, 1983) and promote bronchoconstriction and vascular permeability in the skin (Gerrard, 1985). Research has not shown a clear role for LTE_4. Further research on LTC_4, LTD_4, and LTE_4 may indicate that these compounds have functions similar to LTB_4. For example, the ability to elicit fibroblast migration to the wound site.

Cell Influx - Associated with acute inflammation is the recognition of tissue damage by circulating leukocytes, platelets, lymphocytes and macrophages. Cellular influx to the inflammatory site involves the movement of leukocytes and monocytes from the center of the blood vessel, followed by adhesion to the vascular endothelium (margination). Next, there is movement through the vascular wall and connective tissue (diapedisis), ending in the migration by leukocytes and monocytes in response to chemoattractants released from the inflammatory site. These cells invade the wound site in a defined sequence. Polymorphonuclear leukocytes arrive first, followed by mononuclear leukocytes, fibroblasts and finally endothelial cells (Ross, 1968). Any antigens present at the wound site are engulfed by macrophages and presented in a recognizable form to B and T lymphocytes. Upon stimulation, macrophages produce interleukin 1, which stimulates T-lymphocytes to produce interleukin 2. Interleukin 2 stimulates lymphocytes to differentiate into subsets of helper, suppressor, and plasma cells. Plasma cells then migrate to the area and produce

antibodies that neutralize toxins, cytolyze bacterial wall membranes (cytolytic antibodies), and elicit hypersensitive reactions. Therefore, antigen stimulation results in lymphocyte production of lymphokines, which are chemoattractants for neutrophils (Altman, 1978), monocytes (Lett-Brown, 1976), fibroblasts (Postlethwaite et al., 1976) and other lymphocytes (Ward, 1975). Neutrophils are also attracted to the wound site where they engulf the antigen-antibody complex into a phagosome and release hydrolytic enzymes as well as O_2-, H_2O_2, and OH·. These radicals are generated by a respiratory burst as a result of cellular activation. Neutrophils upon stimulation produce chemoattractants for neutrophils and macrophages (Zigmond and Hirsch, 1973). Neutrophils do not replicate at wound sites, thus, the release of chemoattractants reamplifies the signal for cellular influx. The influx by macrophages then results in the ingestion of cellular debris and production of a chemoattractant for fibroblasts (Wahl, 1981). At this point, the fibroblasts initiate a reparative phase, whereby they migrate to the wound site in an attempt to reconstruct the connective tissue matrix.

The Repairative Phase

The final phase of the inflammatory response involves the migration of the fibroblasts to the wound site and subsequent synthesis of glycosaminoglycans, proteoglycans, fibronectin, and collagen, resulting in the appearance of scar tissue. Attachment to a substratum is an important

step in this response and fibroblasts require fibronectin for both chemotaxis and adherence (Gauss-Mueller et al., 1980). Fibronectin is a glycoprotein consisting of two similar polypeptide chains which are linked at their carboxy (COOH) terminus by disulfide linkages (Fig. 2). These chains are designated alpha and beta, with the alpha chain being slightly larger. The higher molecular weight of the alpha chain is due to the presence of additional amino acids within the heparin/fibrin COOH terminal region (Sekiguchi et al., 1985). Differences in the molecular weights of fibronectin have been reported and may be due to different degrees of glycosylation (Albini et al., 1983, Sekiguchi et al., 1985). The fibronectin chains may further be subdivided into 3 types of regions due to either their cysteine content, protein folding, or fragmentation patterns (Erickson, 1985). Fibronectin has the ability to bind to such proteins as heparin, fibrin, gelatin, collagen, and actin, as well as cell surfaces (Albini et al., 1983; Pierschbacher et al., 1985; and, Sekiguchi et al., 1985). Scatchard analysis reveals that fibroblasts contain 5.3 x 10^{+6} binding sites per cell for fibronectin. The linearality of the Scatchard plot indicates a single class of receptors with a Kd of 8 x 10-7M (Akiyama and Yamada, 1985). Adherence mediated by fibronectin may be required for the proper attachment and detachment in the microtubule/microfilament based motility apparatus. Fibronectin is secreted by fibroblasts and binds collagen at the collagen binding site located near the fibronectin NH_2

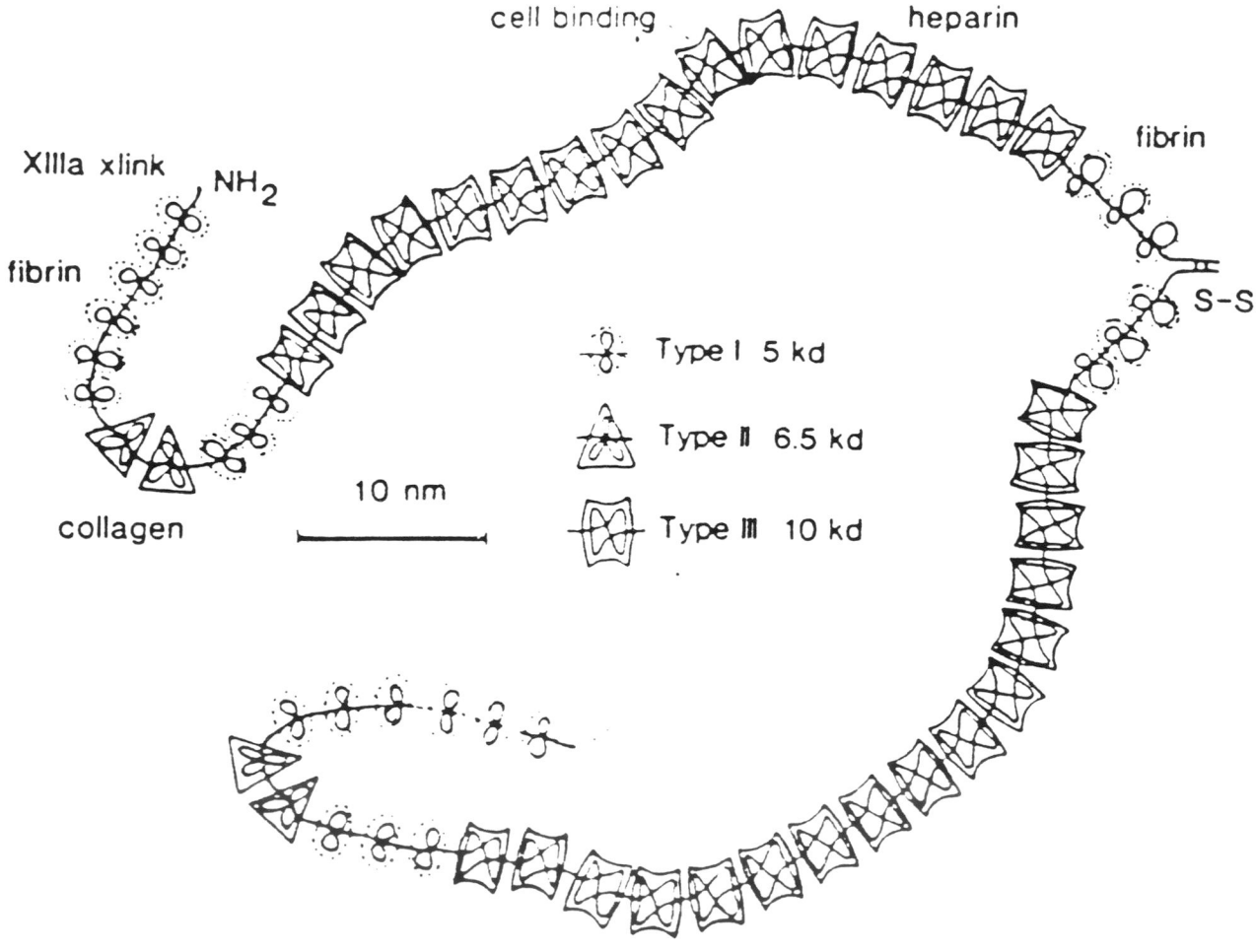
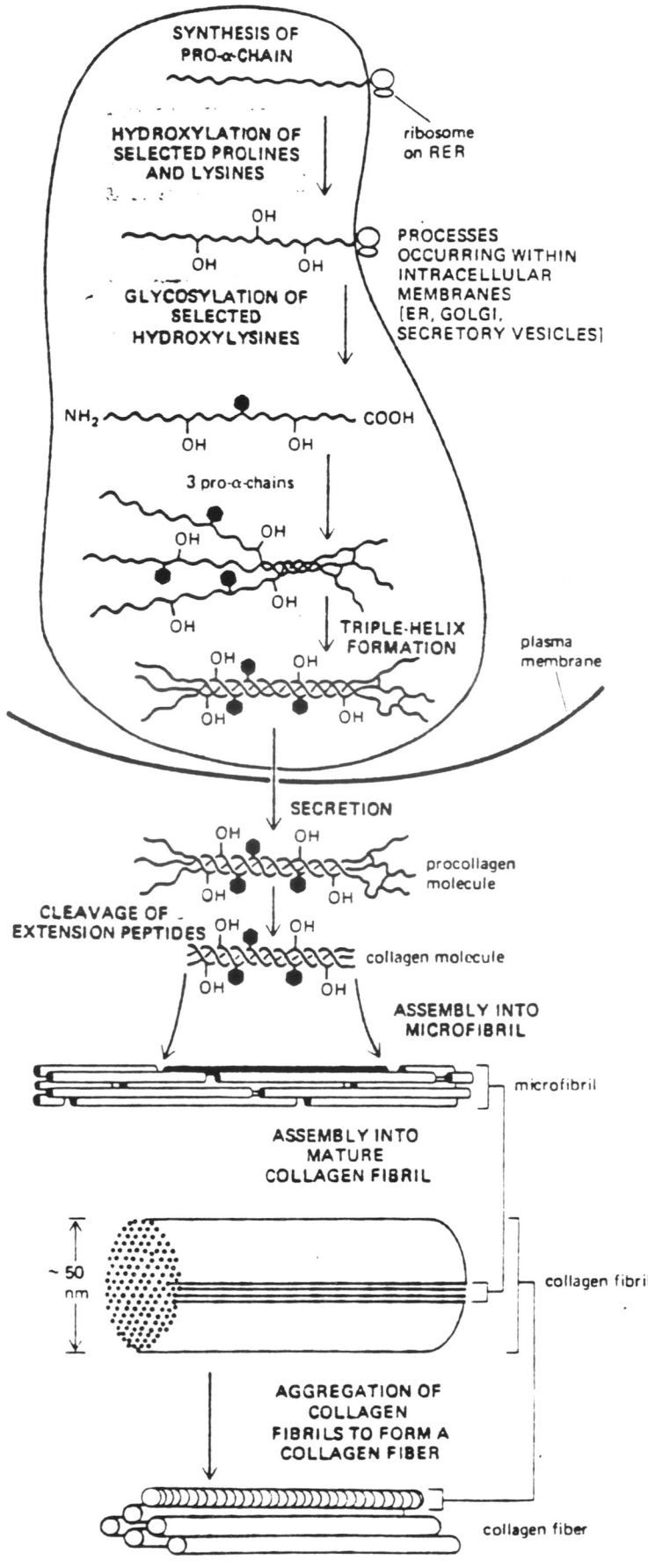


Fig. 2 Fibronectin molecule containing binding regions and the three types of sequence homologies. (Taken from Erickson, 1985).

terminus (Gauss-Mueller *et al.*, 1980). Binding to collagen converts fibronectin into a more extended form, thus enhancing fibronectin binding to other molecules. Fibroblasts then use gangliosides, located in their cell membrane to bind to the fibronectin/collagen complex. The fibroblasts use the carbohydrate moiety of gangliosides to bind to the complex at the cell attachment site of fibronectin, located near the COOH terminus. This cell attachment region may be responsible for 30% of the fibronectin molecule containing a folded B structure (Alexander, 1979). Thus fibronectin, or its fragments generated by tissue damage, may serve in facilitating fibroblast migration as a prerequisite to connective tissue reconstruction.

Initial development and regeneration of connective tissue shows an increase in hyaluronate. This latter disappears with fibroblast proliferation and enhanced collagen synthesis. Collagen is the primary structural element in connective tissue and is made up of three polypeptide alpha-chains wound in a regular helix. The collagen polypeptide chains are synthesized on ribosomes and injected into the lumen of the endoplasmic reticulum as pro alpha-chains, with extension peptides at both the NH_2 and COOH terminus (Fig. 3). In the endoplasmic reticulum each pro alpha-chain combines with two others to form a hydrogen bonded triple stranded helical molecule. The extension peptides aid in guiding this triple helix formation. Along with the assembly of the triple helix is

Fig. 3 Collagen biosynthesis by fibroblasts.
(Taken from Alberts et al., 1983).


SYNTHESIS OF PRO-α-CHAIN
ribosome on RER
HYDROXYLATION OF SELECTED PROLINES AND LYSINES
OH
OH
OH
PROCESSES OCCURRING WITHIN INTRACELLULAR MEMBRANES [ER, GOLGI, SECRETORY VESICLES]
GLYCOSYLATION OF SELECTED HYDROXYLYSINES
NH2
COOH
3 pro-α-chains
TRIPLE-HELIX FORMATION
plasma membrane
SECRETION
procollagen molecule
CLEAVAGE OF EXTENSION PEPTIDES
collagen molecule
ASSEMBLY INTO MICROFIBRIL
microfibril
ASSEMBLY INTO MATURE COLLAGEN FIBRIL
~ 50 nm
collagen fibril
AGGREGATION OF COLLAGEN FIBRILS TO FORM A COLLAGEN FIBER
collagen fiber

the hydroxylation of proline and lysine and post-translational glycosylation of hydroxylysine residues. During secretion, the extension peptides of the procollagen molecule are cleaved off, thus forming tropocollagen. These terminal extension peptides have the ability to regulate collagen deposition by inhibition of collagen synthesis at the level of m-RNA translation. Once formed, tropocollagen associates into microfibrils and microfibrils associate into collagen fibrils. Subsequent crosslinking by extracellular lysyl oxidase forms collagen fibers. Ten to forty percent of all newly synthesized collagen can be eliminated by intracellular degradation via lysosomal enzymes, thus allowing the cell to dispose of imperfect chains. After collagen synthesis there is a remodeling of the connective tissue matrix by collagenase, gelatinase , elastase, and other hydrolytic enzymes. Remodeling is controlled by factors that cause the release, activation, and inhibition of proteolytic enzymes balanced by the synthesis and deposition of matrix proteins and collagen (Kang and Mainardi, 1985).

Fibroblast Chemoattractants

The recognition of damage to the host and subsequent migration to the site of inflammation by the fibroblast is believed to be elicited by the release of two classes of chemoattractants (Table 1). Compounds included in the first class of fibroblast chemoattractants include connective tissue fragments such as collagen and collagen derived

Table 1

List of cell types and their attractants: + (attractant), ? (possible attractant), - (inhibitor). Revised from Schiffman et al., 1983.

	FIBROBLASTS	NEUTROPHILS	MONOCYTES	LYMPHOCYTES	PLATELETS
PDGF	+	?	?	?	+
Plasminogen activators	?	+	+		
HETEs		+	+		
Lymphokines	+	+	+	+	
LTB_4	+	+			
TxA_2	?				+
Fibrin	?				+
Tropoelastin/ elastin	+				
Collagen	+				+
Fibronectin	+				
Complement C5a	+	+	+	?	
Colchicine	-	-	-	-	
Cytochalasin B	-	-	-	-	

peptides (Postlethwaite, 1978), tropoelastin and elastin filaments (Senior et al., 1982), and fibronectin (Gauss-Mueller, 1980). The second class of chemoattractants includes proteins generated by the inflammatory response such as lymphokines (Postlethwaite et al., 1976), and complement 5a [C5a] (Postlethwaite et al., 1979). Complement 5a is a product of the 5th component of complement. Complement 5a contains 74 amino acid (aa) residues with the first 69 aa serving as the recognition site and the last 5 aa at the COOH terminus serving as the active site (Synderman et al., 1981). This glycoprotein is cleaved from the NH_2 terminus of the α chain of C5 during complement activation. Chemotaxis to C5a is diminished by the loss of the terminal arginine by the action of serum carboxypeptidase (Synderman, 1981). Activated platelets, lymphocytes, and neutrophils present at acute inflammatory sites also release such potent fibroblast chemoattractants as platelet derived growth factor [PDGF] (Seppa et al., 1982), lymphokines (Postlethwaite et al., 1976), and leukotriene B_4 (Mensing and Czarnetzki, 1984). All these compounds serve as chemical messengers in eliciting fibroblast chemotaxis to the wound site for initiation of the reparative phase.

Mechanisms of Chemotaxis

Morphological Events - Migration by cells may occur by either random migration, haptotaxis, chemokinesis, or chemotaxis. Random migration is migration in the absence of

any known stimuli, whereas haptotaxis is movement up a gradient due to adhesiveness. Chemokinesis is chemically stimulated, nondirectional motility in the absence of a chemotactic gradient, whereas, chemotaxis is directional motility in the presence of a concentration gradient. Chemotaxis is the most investigated type of motility due to its implication in the inflammatory response. Chemotaxis involves mechanisms of either temporal or spatial sensing (Synderman and Goetzl, 1981; Schiffman, 1983). Temporal sensing involves the perception of the chemoattractant with linear movement for a fixed period of time. This is associated with lag periods where the cell stops, and perceives a new concentration before moving again. Bacteria are prime examples of this mechanism, using one chemoreceptor and memory. Spatial sensing is associated with eukaryotic cells and exhibits alteration of cell shape and morphological orientation toward the concentration gradient. Migration is in a curvilinear fashion without pauses, and with detection of the gradient across the length of the cell via multiple receptors. This type of sensing allows differentiation of concentrations as little as 0.1% across the cell surface (Synderman and Goetzl, 1981). This is accomplished by altering the distribution, number, and affinity of chemotactic receptors. Exposure to a chemoattractant results in polarized elongation of the cell with the appearance of a broad lamellipodium anteriorly. Located at the posterior end of the cell is a thin uropod with terminal arborations. Located beneath the plasma

membrane and within the cell are the cytoskeletal processes. Throughout the cell, are microtubule organizing centers that function as the point of origin for microtubules. Concentrated just beneath the plasma membrane is the actin microfilament network. There is a localization of receptors in the lammellipodia, with vesicle concentration in the uropod. Chemoattractant binding results in the ligand-receptor complex being picked up at the leading edge, followed by transport over the dorsal surface of the cell. Old cell mass is endocytosed at the rear with new cell mass and receptors arising from the Golgi complexes to be reincorporated into the new cell membrane (Nemere et al., 1984). Both Golgi complexes and microtubules are organized in the direction of migration. Cytochalasin B blocks actin filament rearrangement and pseudopod retraction without the loss of orientation. This indicates that microfilaments are critical for the contractile events of directed locomotion and morphological changes. Colchicine inhibits microtubules without altering the formation of pseudopodia, thus indicating their importance in the initiation and stabilization of cellular orientation toward a chemotactic gradient (Synderman and Goetzl, 1981; Schiffman, 1982).

Biochemical Events - Chemoattractant binding also stimulate Ca^{+2} influx both extracellularly and from membrane bound compartments, thereby, increasing the concentration of ionized intracellular Ca^{+2} (Naccache et al., 1977). Calcium release is accomplished by an increase in permeability to Na^{+} through membrane potential insensitive channels,

resulting in H^+ leaving the cell and subsequent membrane polarization. A pH change also arises and Ca^{+2} is released. These events elicit activation of a membrane bound Na^+-K^+ ATPase. A calcium influx activates PA_2 resulting in the production of PGs, LTs, and Hetes, each with the ability to reamplify the response in other cells. Increases in free ionized Ca^{+2} may activate a tyrosine specific kinase, a c-AMP kinase or calmodulin. A specific kinase may be needed to phosphorylate a chemotactic regulatory protein known as lipocortin. Recent research indicates that lipocortin acts as a regulatory protein in chemotaxis (Hirata, 1980). This is due to the fact that the phosphorylated form of lipocortin has the ability to inhibit chemotaxis by binding to PA_2 (Blackwell *et al.*, 1980; Hirata, 1980). Associated with chemotaxis, is a brief increase in c-AMP. However high levels of c-AMP inhibit chemotaxis, while high levels of c-GMP enhance chemotaxis (Tse *et al.*, 1972; Rivkin *et al.*, 1974). This may be explained by the following mechanism. Attractant binding followed by Ca^{+2} influx and c-AMP production may stimulate a c-AMP dependant kinase and calmodulin. The c-AMP dependant kinase may serve to phosphorylate lipocortin at tyrosine residues. Binding of Ca^{+2} by calmodulin then results in a conformational change and enzyme activation. Once activated, calmodulin may accelerate phosphodiesterase activity resulting in termination of the c-AMP response and elevation of guanosine triphosphate levels. Guanosine triphosphate may then induce conformational changes in the 6S tubulin dimers resulting in

polymerization of microtubules from microtubule organizing centers. Motility is then generated by the polymerization-depolymerization of microtubules or by a sliding mechanism through ATP hydrolysis by dynein arms. Free ionized Ca^{+2} also stimulates microfilament mediation of chemotaxis. The mechanisms of motility may be very similar to the mechanisms of sliding actin/myosin filaments in muscle. However, in migrating cells the actin/myosin complex is three dimensional, and regulated by an actin binding protein (ABP) and gelsolin (Stendahl et al., 1980). Increases in actin network structure by ABP enhances the contractility of actin by myosin. The ABP crosslinks actin to form an actin lattice with interspersed myosin filaments, thus providing a contracting mass. Gelsolin divides actin filaments in the presence of high Ca^{+2} by shorting and severing actin filaments between points of crosslinking. The actin lattice moves from high concentrations of Ca^{+2} to low concentrations of Ca^{+2}. Free actin filaments interacting with myosin move away from domains of decreased crosslinking because of the increased efficiency of contraction in the more crosslinked domains. Therefore, any part of the cell attached to the lattice would move accordingly. Calmodulin would then play a role in the events leading up to the phosphorylation of myosin light chains, and subsequent motility. The ATPase activity of the myosin head group would then generate the energy for the sliding mechanism between the actin and myosin filaments.

Fibrotic Diseases and their Relationship with the Leukotrienes and Thromboxane A_2

The function of the acute inflammatory response is to complete healing with restoration of normal tissue architecture and function. Any breakdown in this process may result in little to no attempt at wound healing with resulting fibrosis and excessive connective tissue formation. This chronic inflammatory response may be due to the continuation of the initial inflammatory stimulus. This continuation leads to enhanced tissue destruction by proteases, as well as elevated radical formation, AA metabolism, and cell influx. Released proteases have the ability to modify proteins at the inflammatory site with subsequent antibody formation against this "self antigen". Enhanced AA metabolism and leucocyte influx may generate such oxidative radicals as OH·, H_2O_2, and O_2-. These radicals are generated either by endoperoxide formation or by respiratory bursts in activated leukocytes. Thus excessive AA metabolism may generate radicals that attack surrounding tissue, or act as chemoattractants in stimulating cellular influx. These events may result in the appearance of many pathological disorders including rheumatoid arthritis, breast cancer, and many types of pulmonary fibrotic diseases. The pathogenesis of these diseases seems to indicate that the generation of chemoattractants such as the LTs and TxA_2 serve to elicit fibroblast migration to the afflicted site resulting in

excessive fibrotic buildups. Examination of a few of the diseases associated with excessive fibroses follows.

Rheumatoid Arthritis - Rheumatoid arthritis is characterized by high percentages of neutrophils and elevated levels of LTs within synovial fluid. As the disease progresses, fibrotic involvement is encountered. It is interesting to note that patients with severe arthritis have been shown to contain antibodies for the protein lipocortin (Hirata, 1981). Lipocortin inhibits phospholipase activity *in vitro*. Studies postulate that a c-AMP dependent kinase phosphorylates the lipocortin inhibitor (Hirata *et al.*, 1981), resulting in the inactivation of lipocortin and initiation of AA metabolism. Thus, prolonged inactivation of lipocortin may result in excessive chemotaxis by neutrophils and fibroblasts and an overproduction of LTs. The released LTs may possibly elicit the attraction of more neutrophils and fibroblasts to the afflicted area.

Breast Cancer - Diffuse fibrotic buildup is also associated with many forms of breast cancer. It has been shown that tumor cells obtained from patients with breast cancer secrete a potent chemoattractant for fibroblasts (Gleiber and Schiffman, 1984). Research has also shown that breast tumor cells treated with ionophore A-23187 and L-cysteine produce LTs (Nelson *et al.*, 1982). Thus, fibroblasts may migrate to tumor cells due to a chemoattraction for both the breast tumor cells and possibly

the LTs. This migration to the tumor cells is followed by fibroblast proliferation and subsequent formation of dense fibrotic masses.

Progressive Systemic Sclerosis and other Fibrotic Diseases - Progressive systemic sclerosis is another example of a disease characterized by diffuse fibrosis. The primary event in this disease is postulated to be endothelial cell injury in blood vessels (Kang and Mainardi, 1985). The increased cell damage produces interstitial edema, fibroblast stimulation, and fibrosis. Two more examples of fibrotic diseases encountered by clinicians is diffuse interstitial fibrosis and idiopathic fibrosis. It is believed that the local production of specific chemotactic factors recruits inflammatory and immune effector cells into the alveolar interstitium (Kang and Mainardi, 1985). These chemotactic factors may prove to be LTs released from leukocytes and/or thromboxane A_2 released from platelets. As a consequence, this inflammatory response results in extensive damage to lung connective tissue, and endothelial membranes. Following this damage, extensive fibroses may be encountered.

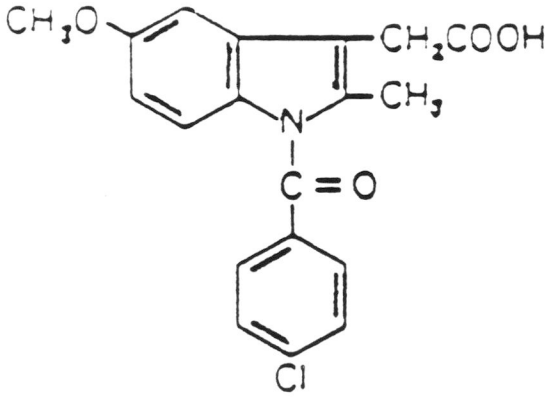
Despite the high occurrence of these diseases, medical therapy is not curative and often unsatisfactory and ineffective. Thus, our understanding of their pathogenesis is incomplete. Excessive fibrosis is often associated with an overproduction of AA metabolites, therefore inhibitors of their synthesis or recognition may prove to be of therapeutic value in the treatment of many fibrotic

diseases. One mechanism of treatment may be through the inhibition of AA metabolism by glucocorticoids or non-steroidal anti-inflammatory agents.

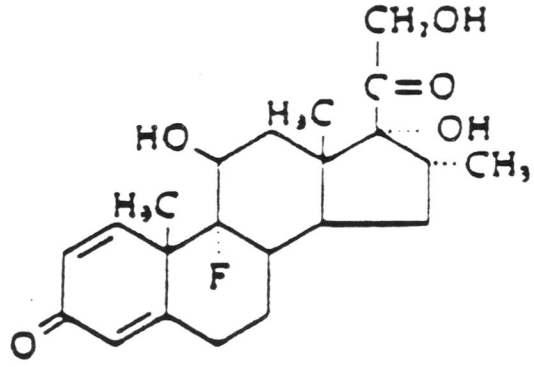
Anti-Inflammatory Agents

Indomethacin - Indomethacin (Fig.4) is a non-steroidal anti-inflammatory agent with the ability to inhibit granuloma formation, edema, pain, and PG synthesis. Indomethacin inhibits PA_2 at very high doses and cyclo-oxygenase at a concentration of 14.3 ng/ml. Indomethacin is classified as a substrate competitive irreversible inhibitor (Kulmancz, 1983). This compound competes with AA for cyclo-oxygenase but does not covalently bind to the enzyme. The active site of indomethacin contains an -methyl acetic acid side chain, which binds to contours on cyclo-oxygenase at sites which accommodate large groups of aryl acetic acids (Shen and Winter, 1977). Indomethacin has also been shown to suppress leukocyte influx (Blackham 1979) as well as PG formation. Therefore, indomethacin may serve as an important mediator of both the inflammatory response and cell migration.

Glucocorticoids - Dexamethasone (Fig. 4) is an optically active glucocorticoid with the ability to induce hyperglycemia followed by secondary hyperinsulinimia. Glucocorticoids increase the degradation and decrease the synthesis of fat, proteins, DNA, and RNA. Glucocorticoids also have the ability to inhibit lymphokine production and PA_2 (Hirata, 1983; Munck 1984). Recently it has been found



a. Indomethacin



b. Dexamethasone

Fig. 4 Structures of a.) indomethacin and b.) dexamethasone

that glucocorticoids inhibit both the expression of Fc receptors and tumoricidal activity of macrophages (Munck et al., 1984). Dexamethasone rapidly penetrates the cell due to its hydrophobic nature and binds reversibly to its cytosolic receptor to form a non-covalent complex of high affinity.

Glucocorticoid Receptor - The glucocorticoid receptor is a single polypeptide with 5,000 to 50,000 sites per cell. It has a molecular weight of 85-95 Kd containing three distinct domains; a steroid binding domain, a DNA binding domain, and an immunoactive domain (Rousseua, 1984). Steroid binding increases the affinity of the receptor for sites in the cell nucleus. Binding of the receptor to the steroid is second order with disassociation of the complex being first order (Baxter, 1976). The steroid binding domain is rich in lysine and arginine residues which bind the steroid. This binding is inhibited by proteases and sulfhydryl reagents. The steroid binds the receptor when the receptor is phosphorylated and reduced, thus requiring the availability of thiol groups. A thioredoxin associated factor prevents the exposure of basic aa located on the DNA binding subunit. This prevents binding to DNA by the inactivated receptor. Molybdate protects reduced thiol groups on the receptor which are essential for steroid binding. Steroid binding initiates dephosphorylation and release of the thioredoxin associated factor with dissociation of the receptor into monomeric units in order to allow DNA binding. Binding of the complex to nuclear

receptors initiates transcription and translation with subsequent protein synthesis. The receptor is then recycled in the cytoplasm, reduced and rephosphorylated in preparation for subsequent binding.

Lipocortin - Lipocortin (M.W. 40 Kd) is found in both untreated and dexamethasone treated cells. Treatment of cells with dexamethasone rapidly increases the supply of this protein in the cell membrane. Lipocortin reduces the Vmax but not the Km of PA_2, thus forming stoichiometric complexes (Hirata, 1983). Lipocortin inhibits this enzyme by binding to the hydrophobic PA_2 Ca^{+2} binding site. This is substantiated by the ability of lipocortin to bind four moles of Ca^{+2} (Hirata, 1983). Phosphorylation of lipocortin results in a loss in the ability to inhibit phospholipase A_2 and chemotaxis (Hirata, 1980, 1981). Studies indicate that chemoattractant binding induces a Ca^{+2} influx which activates PA_2 and the phosphorylation of lipocortin. Further research needs to be conducted to determine if phosphorylation is by a Ca^{+2} kinase, tyrosine dependant kinase, or cAMP dependant kinase

PROPOSED RESEARCH

The purpose of this study was to determine the effect of inhibitors of AA metabolism on fibroblast migration. Inhibitors were selected for their ability to inhibit PA_2 activity and cyclo-oxygenase. Inhibitors of AA metabolism may be grouped into two classes; nonsteroidal anti-inflammatory agents and glucocorticoids. A representative of each class was examined to determine its effect on fibroblast chemotaxis. Indomethacin represented the nonsteroidal anti-inflammatory agents, while dexamethasone represented the glucocorticoids. Indomethacin directly inhibits cyclo-oxygenase activity. Whereas, glucocorticoids inhibit PA_2 through the induction of lipocortin (Blackwell et al., 1980; Hirata, 1981). This study examined the effects of these anti-inflammatory compounds on fibroblast chemotaxis towards conditioned medium.

MATERIALS AND METHODS

Cell Culture Techniques - Human fetal foreskin fibroblasts (Earl Clay Co., Norcross, GA) were grown to confluency in 25 cm^2 tissue culture T-flasks containing medium 199 (Sigma, St. Louis, MO) supplemented with 8% fetal calf serum [FCS] (Sigma, St. Louis, MO), and 1% penicillin/streptomycin. Propagation of the cells consisted of removal of the maintenance media followed by three washes with Hank's buffer (pH 7.2) consisting of 0.14M NaCl, 5mM KCL, 1.5mM KH_2PO_4, 15mM $NaHCO_3$, and 0.7mM Na_2HPO_4-$7H_2O$. Fibroblasts were harvested by a five minute trypsinization procedure utilizing a solution of 0.016% trypsin (Diffco, Detroit, MI.) and 0.09% EDTA in Hank's buffer. Trypsinization was then terminated by the addition of maintenance medium to the T-flasks. Cells were seeded at a concentration of $1x10^{+5}$ cells/ml and maintained at a temperature of 37.5 ^{o}C with 95% O_2 and 5% CO_2. Cells from the 10th to 15th passages were used for this study.

Isolation of Conditioned Medium - Fibroblasts were harvested as described, adjusted to a concentration of $3.0 \times 10^{+6}$ cells/ml in Medium 199 with 8% FCS and grown to confluency. Attempts at growing cells to confluency utilizing a modified defined medium (see below) revealed poor cell viability. The maintenance medium was decanted and the cells were washed three times to remove any trace of FCS. Cells were then incubated with a modified defined medium consisting of Medium 199 supplemented with thyroxine

(10 mg/ml), insulin (5 ug/ml), transferrin (5ug/ml), selenium (5 ug/ml) [Collaborative Res. Inc., Lexington, MA], and 1% penicillin/streptomycin. After 3 days, the conditioned medium was removed and centrifuged at 4,000g for 10 min. at 4°C. The resulting supernatant was removed and stored at -40°C for subsequent assays.

Partial Characterization of the Conditioned Medium. - Conditioned media was subjected to sodium dodecyl sulfate (SDS) 7.5, 10, and 12% polyacrylamide gel electrophoresis as described by Laemmli (1970). A one hundred microliter quantity of sample was suspended in a 100 ul cocktail containing a 1.4% Tris stacking buffer with 2% SDS, 10% glycerol and 5% bromophenol blue. Samples were then loaded into respective gels, and electrophoresed at 12.5 mA per gel at a constant current. Protein bands were visualized by staining with either a Coomassie Blue or Silver Nitrate stain. For Coomassie Blue staining, gels were fixed in 50% methanol and 7.5% acetic acid for 4 to 6 hours. Gels were then stained with Coomaasie Blue (0.25%) for 15 min. Destaining consisted of repeated immersions of the gels in 15% methanol and 7.5% acetic acid. Silver Nitrate staining of gels consisted of a 45 min. fix in 6% glutaraldehyde. Gels were then stained with 0.9% $AgNO_3$, and developed in a solution containing 0.005% citric acid and 0.02% formaldehyde. Proteins contained in the conditioned media were compared with high molecular weight standards (BioRad, Richmond, CA), 0.250 ug/ml Type I Collagen (Flow Labs, McLean, VA), fibronectin (Sigma, St. Louis, MO,

Collaborative Res. Inc., Lexington, MA), and defined medium. A LKB ultrascan XL laser densitometer was utilized for gel scans.

Chemotactic Assays - Polycarbonate 15mm filters (Nucleopore Corp., Pleasanton, CA) were placed in chemotaxis filter baskets (East Carolina University Physics Shop, Greenville, NC) and washed with 0.5% acetic acid for 20 min. at 60 °C. Filters were then rinsed with distilled H_2O and immersed in a solution containing 0.0025% gelatin (Sigma, St. Louis, MO) for 60 min at 100 °C. Filters were dried at 37.5 °C overnight. Two hundred microliters of conditioned medium was added to the bottom well of modified Boyden chambers. Gelatin coated filters were placed on each well containing the test solution. Initial cell harvesting procedures were preformed as described by Gleiber (1985). In initial indomethacin studies, T-flasks containing confluent fibroblasts were washed twice with Hank's buffer and incubated with 0.025% trypsin/EDTA for 10 min. at 37.5 °C. Modification of this procedure for dexamethasone studies involved washing the cells twice for 1 min. in Hank's buffer. Cells were then washed with 0.025% trypsin/EDTA for 10 seconds. The supernatant was discarded and the cells were allowed to incubate in the trypsin backwash for 15 min. at 37.5 °C. In all experiments, Medium 199 containing 8% FCS was added to the T-flasks in order to inactivate the trypsin. Cells treated by the backwash method displayed a two fold increase in cell response to all treatments. Therefore, later studies utilized this method

for cell isolation. After inactivation by trypsin, the cell suspension was centrifuged at 150g for three min. The supernatant was removed and cells were resuspended in Medium 199 containing 2.5 ug/ml bovine serum albumin (BSA). Cells were then adjusted to a concentration of 3.5 x 10^{+5} cells/ml. A 500 microliter aliquot of the cell suspension was added to the upper half of the Boyden chamber. Thus, the filter separated the cell suspension which was in the top from attractant which was in the bottom half of the chamber. Chambers were incubated for 4 hours at 37.5 °C in an atmosphere of 5% CO_2 and 95% O_2. After 4 hours of incubation, the filters were removed, fixed in 100% isopropyl alcohol, and stained with a May Greenwald/Giemsa stain. The percent cell migration through the filter depended on the number of nuclei on the bottom of the filter which was in contact with the chemoattractant (see Appendix I). Nuclei in an area of $1mm^2$ (1/12th of the total area of migration) were microscopically counted to quantitate results. Experiments were run in duplicate and data was subjected to a Student's t-test, analysis of variance, and Schiffe's test. Significance was determined at a confidence level of 0.05.

A preliminary study was conducted in order to determine the chemotactic activity of conditioned medium . The cell suspensions were obtained as described and added to the upper half of Boyden chambers. The lower wells of the chambers contained either: Medium 199, Medium 199 supplemented with 8% FCS, Modified Medium 199, or

conditioned medium.

Subsequent inhibition studies utilized conditioned medium (CM) as a chemoattractant. Indomethacin (Sigma, St.Louis, MO) and dexamethasone (Sigma, St. Louis, MO) were solubilized at 1 x 10^{-2}M in 100% ETOH. These stock solutions were diluted to concentrations ranging from 1 x 10^{-4} to 1 x 10^{-10}M in Medium 199. Fibroblast suspensions were exposed to concentrations of inhibitors ranging from 1 x 10^{-4} to 1 x 10^{-10}M for 25 min. at 37.5 ^{o}C. Positive controls contained conditioned medium and no inhibitors, while negative controls contained no inhibitors with Media 199 in the lower well. Indomethacin (10^{-4}M) and dexamethasone (10^{-10}M) were also examined for chemotactic ability. Inhibitors were adjusted to their respective concentrations in Medium 199 and added to the lower half of Boyden chambers. Similar experiments involved adding cells exposed to inhibitors to the upper half of the Boyden chambers with lower chambers preloaded with Medium 199. Experiments were then performed as described.

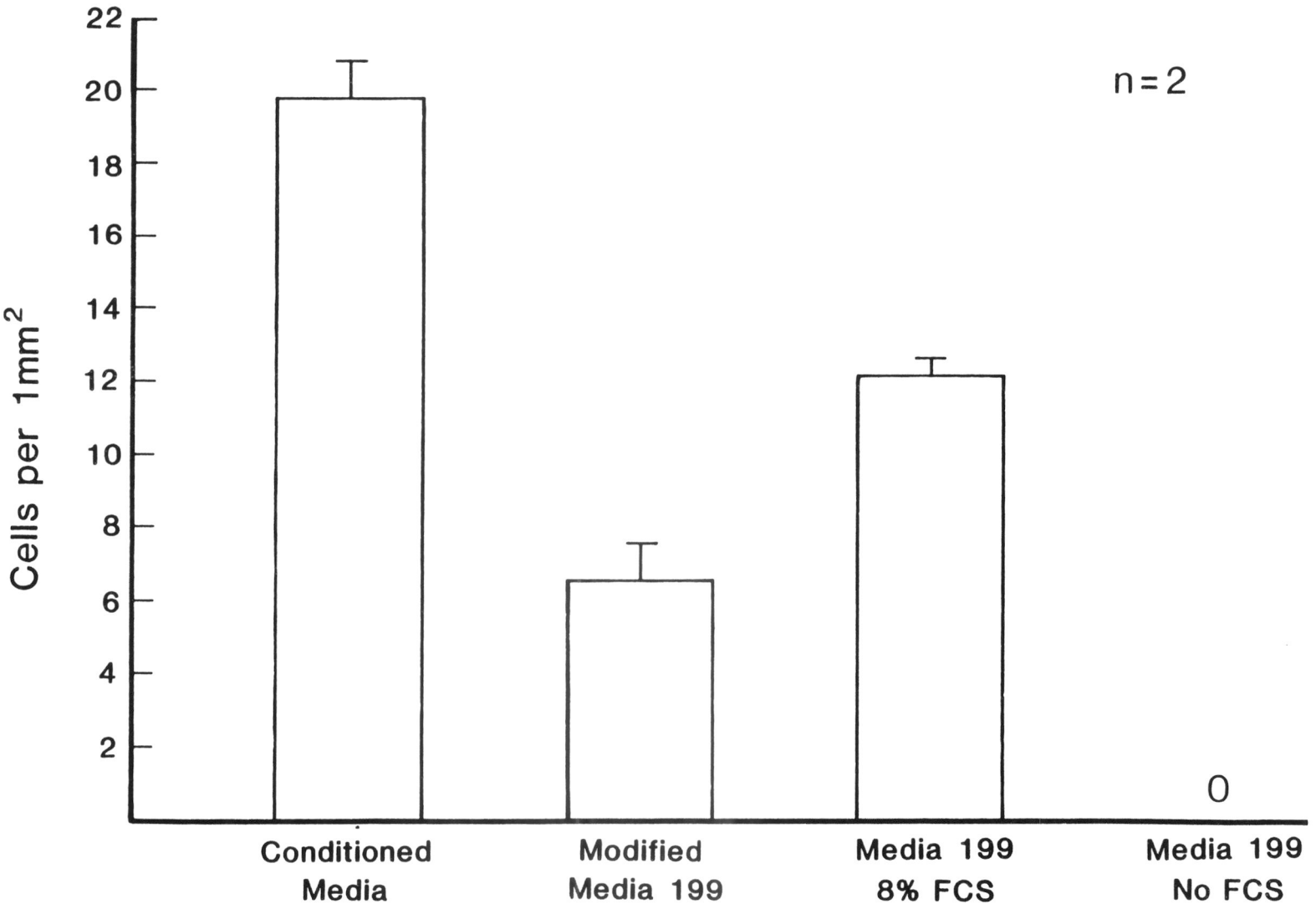
RESULTS

Conditioned Medium - Medium 199 elicited no fibroblast chemotaxis (Graph 1). Supplementing this medium (modified media, MM) with thyroxine, insulin, selenium and transferrin resulted in the appearance of chemotactic activity (6.3 2.6 cells/mm^2). Addition of 8% FCS to Medium 199 also resulted in enhanced chemotaxis (12.25 1 cells/mm^2). The highest chemotactic activity resided in the MM which had been exposed to fibroblasts for three days (19.5 2.5 cells/ml). This CM was then utilized for all subsequent assays.

A 7.5% polyacrylamide gel electrophoresis of both MM and CM revealed a 73 Kd protein (Fig. 5), with CM containing an additional 60 Kd protein. Type I collagen revealed a 210, 130, and 120 Kd protein corresponding to the one alpha 2 and two alpha 1 polypeptide chains comprising this glycoprotein. Fibronectin obtained from Sigma revealed two faint 230 and 220 Kd proteins corresponding to the alpha and beta chains of fibronectin. Fibronectin from Sigma also contained a 175, 150, 137 and 60 Kd protein. Identical proteins with weights of 230, 220, 175, and 150kd were obtained with fibronectin from Collaborative Research. Samples were then subjected to electrophoresis utilizing higher concentrations of acrylamide, in order to determine lower molecular weight proteins. A 10% (Fig. 6) and 12% (Fig. 7) acrylamide gel revealed the appearance of an additional 70 Kd and 28 Kd protein in the CM. A silver stain of the 10% acrylamide gel (Fig. 8) was subsequently employed to enhance faint staining bands in both fibronectin

Graph 1.- Total number of fibroblasts migrating per $1mm^2$ in response to various types of media. Cells were prepared as described in materials and methods.

Fibroblast Chemotaxis Toward Conditioned and Defined Media



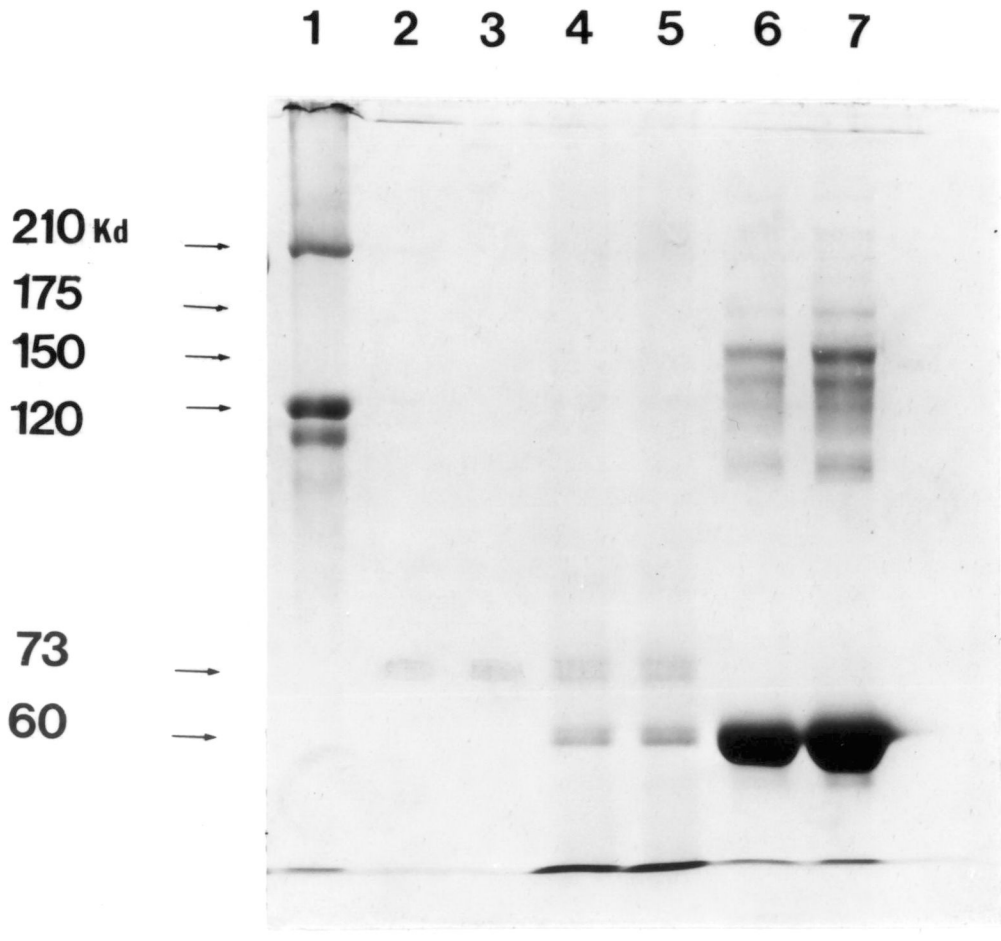


Fig. 5 7.5% SDS electrophoresis: Lane 1, Collagen: Lane 2 and 3, MM: Lane 4 and 5, CM: Lane 6 and 7, Fibronectin.

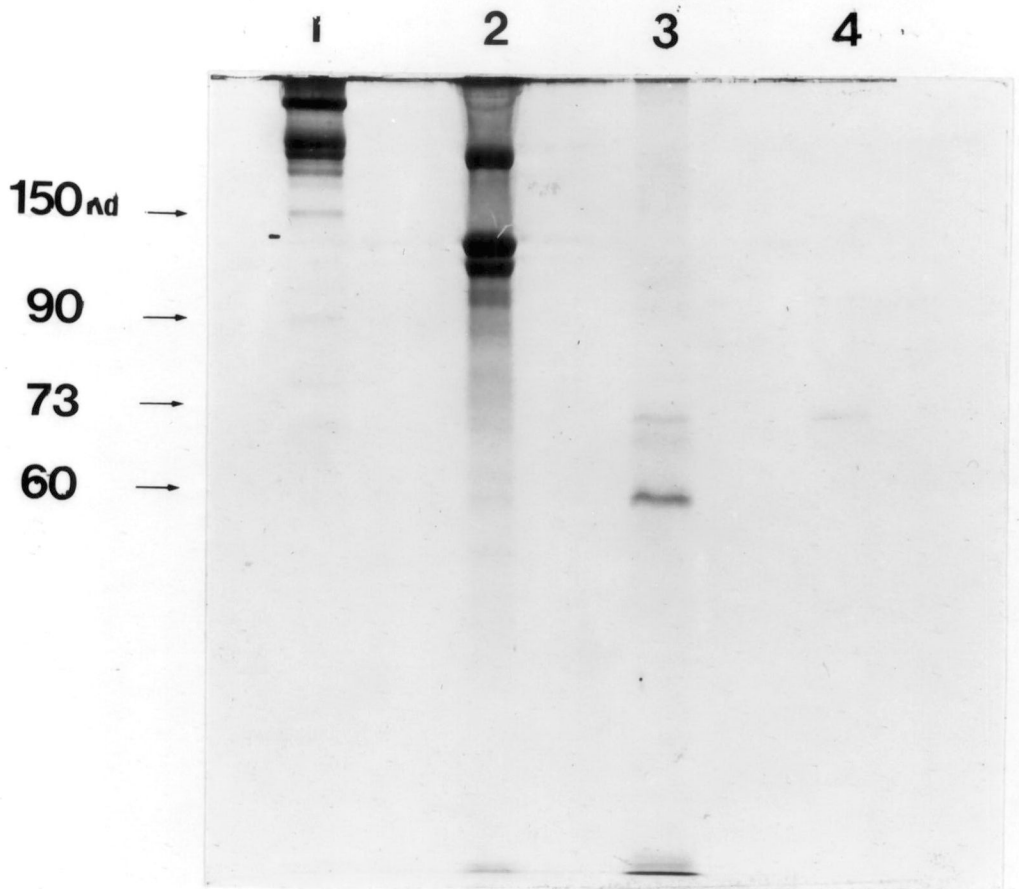


Fig. 6 10% SDS electrophoresis: Lane 1, Fibronectin: Lane 2, Collagen: Lane 3, CM: Lane 4, MM.

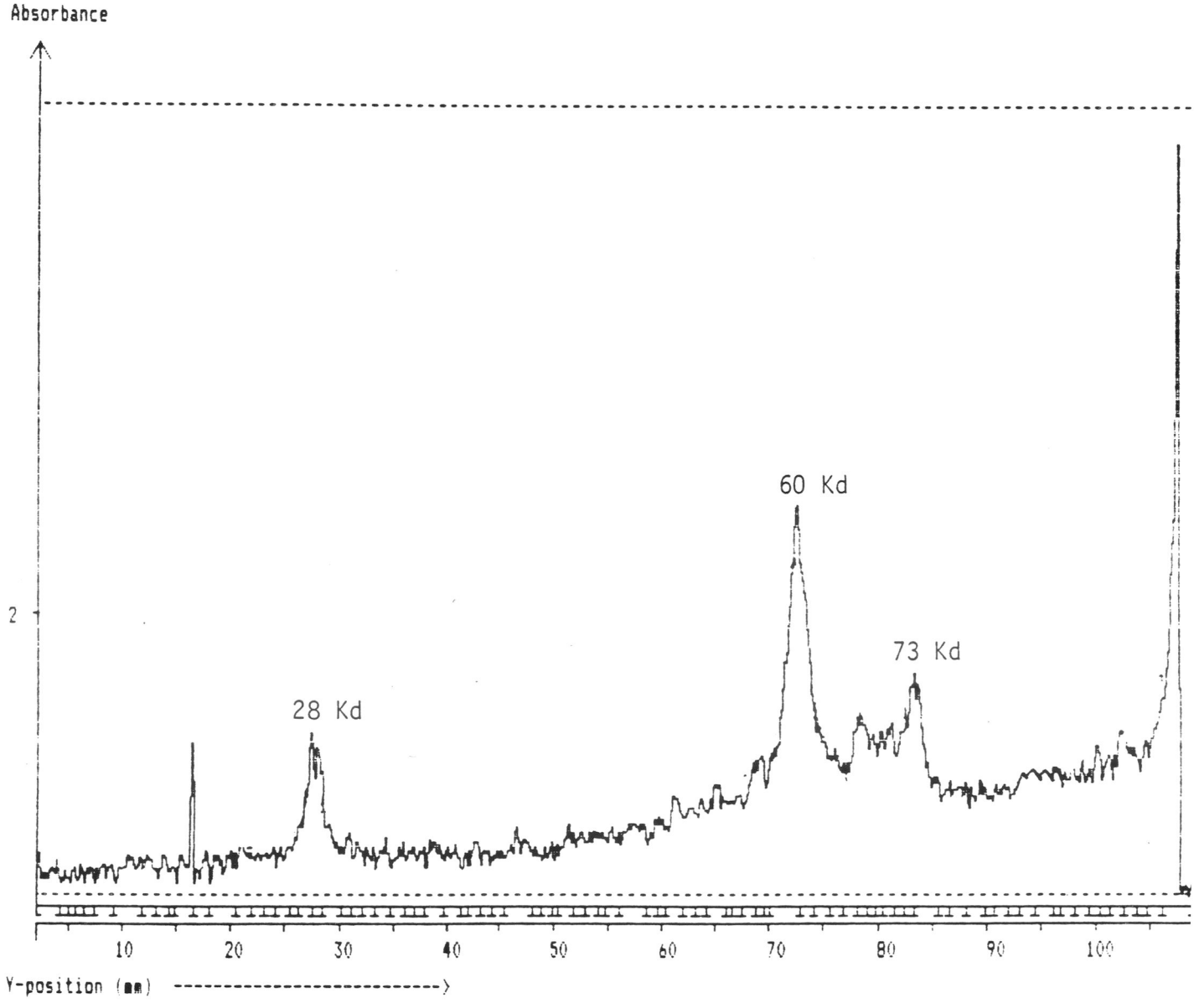


Fig. 7 A Densitometer scan of a 12% gel containing CM.

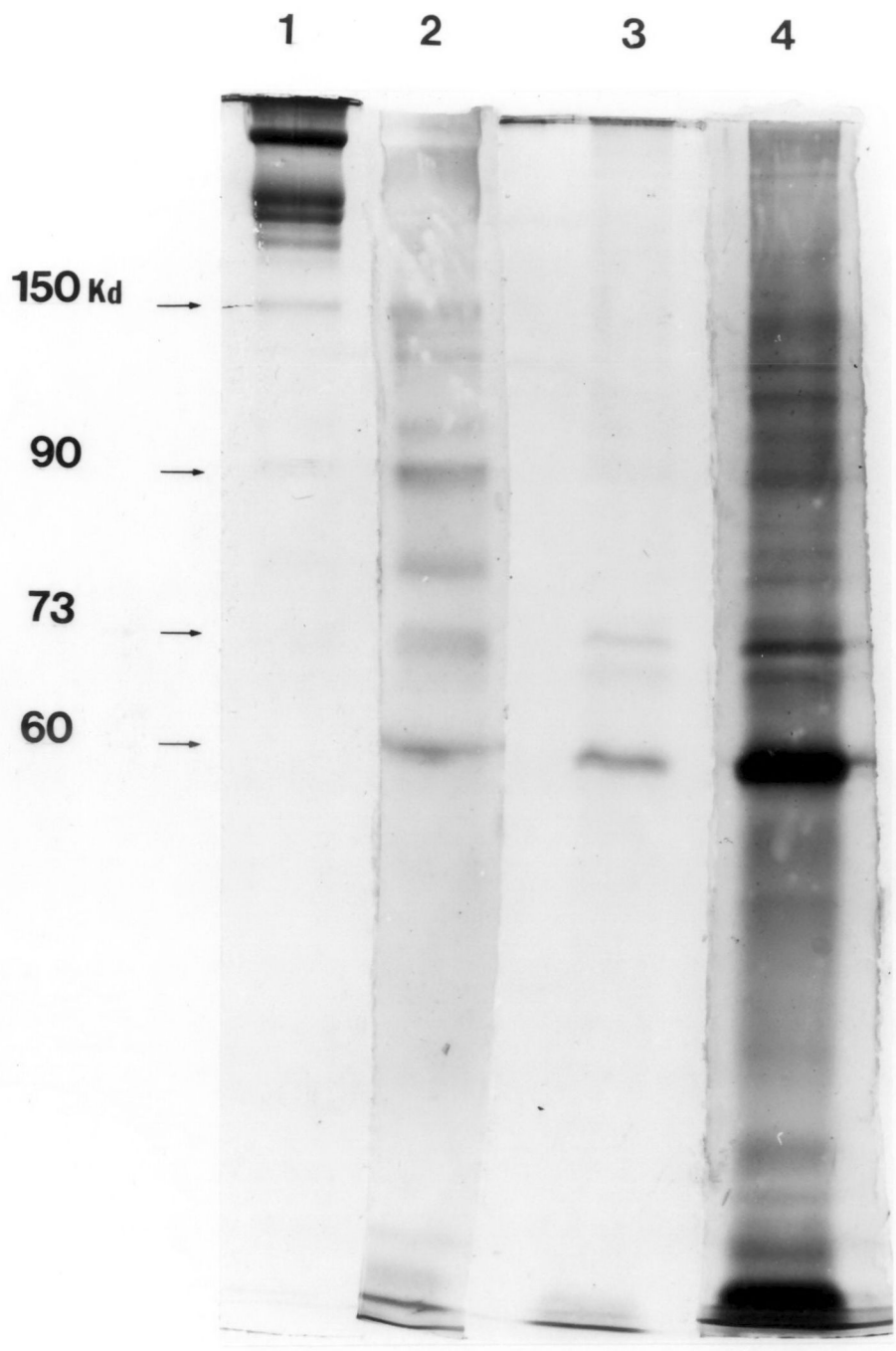


Fig. 8 10% SDS electrophoresis utilizing either a Coomassie Blue or $AgNO_3$ stain: Lane 1, Coomassie Blue stain of Fibronectin; Lane 2, $AgNO_3$ stain of Fibronectin; Lane 3, Coomassie stain of CM; Lane 4, $AgNO_3$ stain of CM.

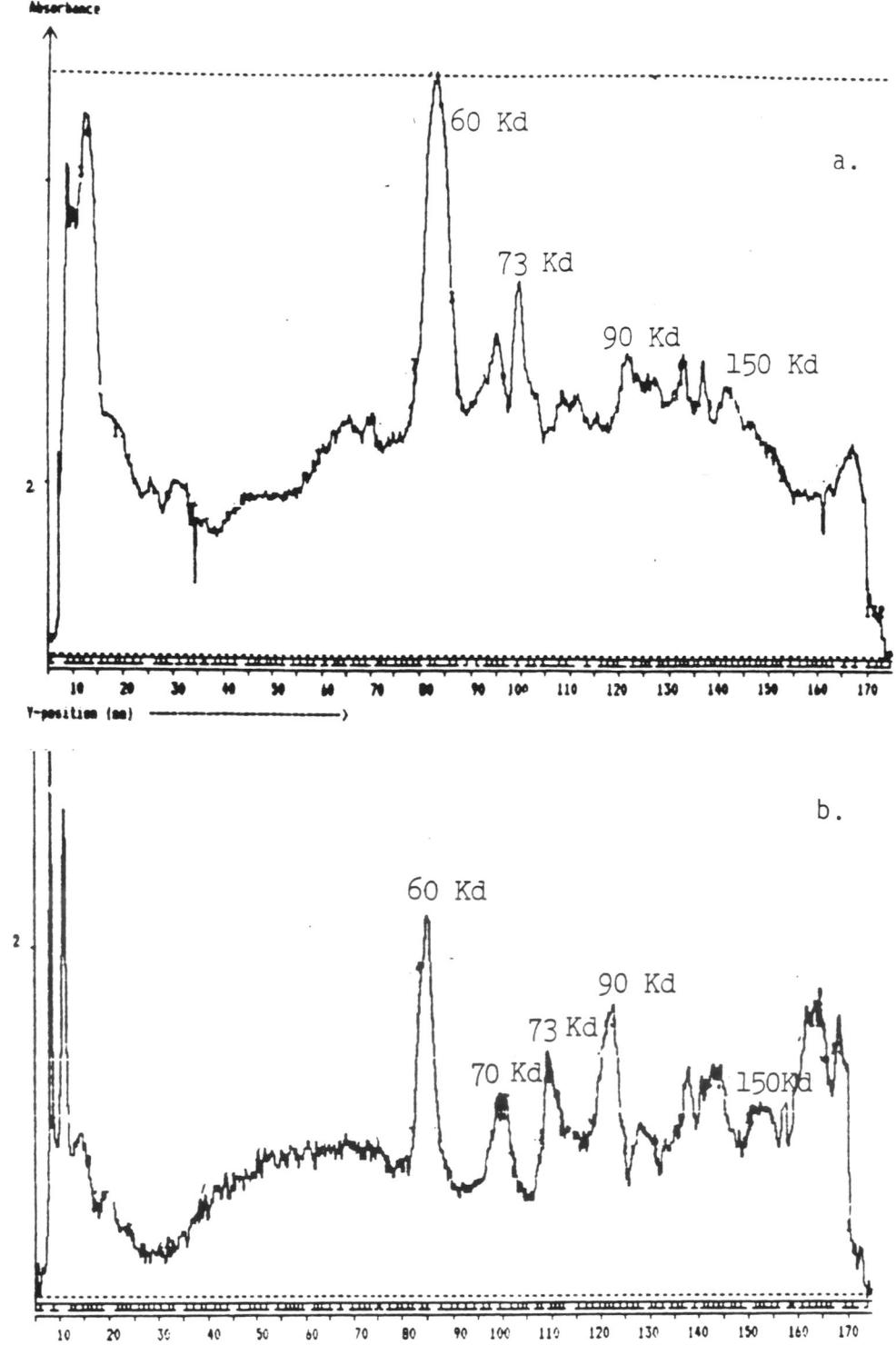


Fig. 9 A Densitometer scan of a 10% SDS silver nitrate stained gel containing a.) CM and b.) Fibronectin

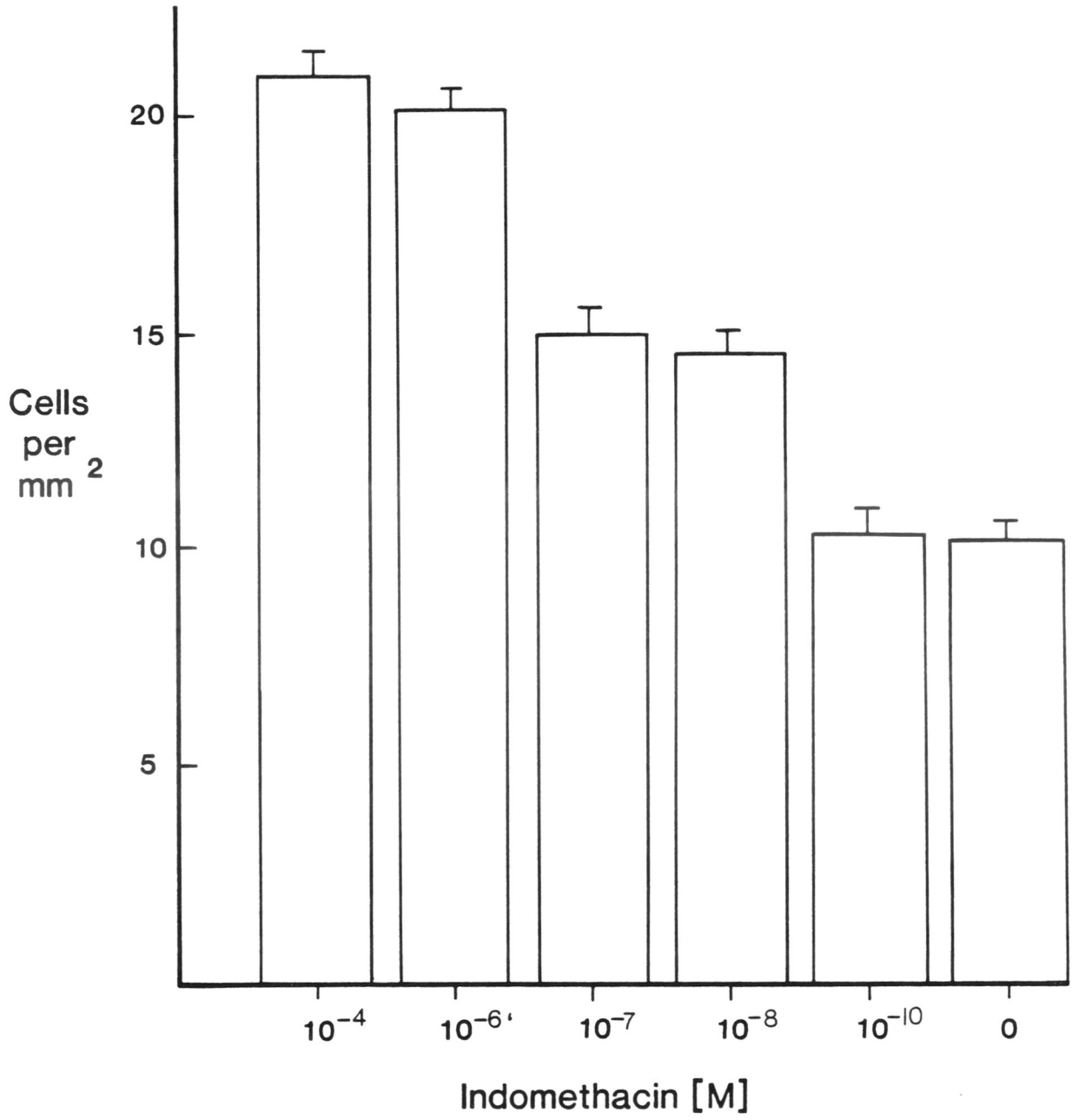
and CM. This method revealed an additional 137 and 90 Kd proteins in CM, and 90, 80 and 60 Kd proteins in fibronectin.

Inhibition Studies - Fibroblasts treated with 1x10-4M indomethacin revealed a 91% increase in migration over controls (Graph 2). There was also a 79% increase in chemotaxis in cells treated with 1 x 10^{-6}M indomethacin. This effect diminished with decreasing concentrations of indomethacin. A Student's t-test (a=0.05) revealed no significant difference between indomethacin treated cells at 1 x 10^{-8}M to 1 x 10^{-10}M and positive controls containing conditioned medium. Indomethacin placed in the bottom of Boyden chambers failed to stimulate cell migration. Indomethacin placed in the upper halves of Boyden chambers failed to stimulate cell migration in the absence of conditioned medium.

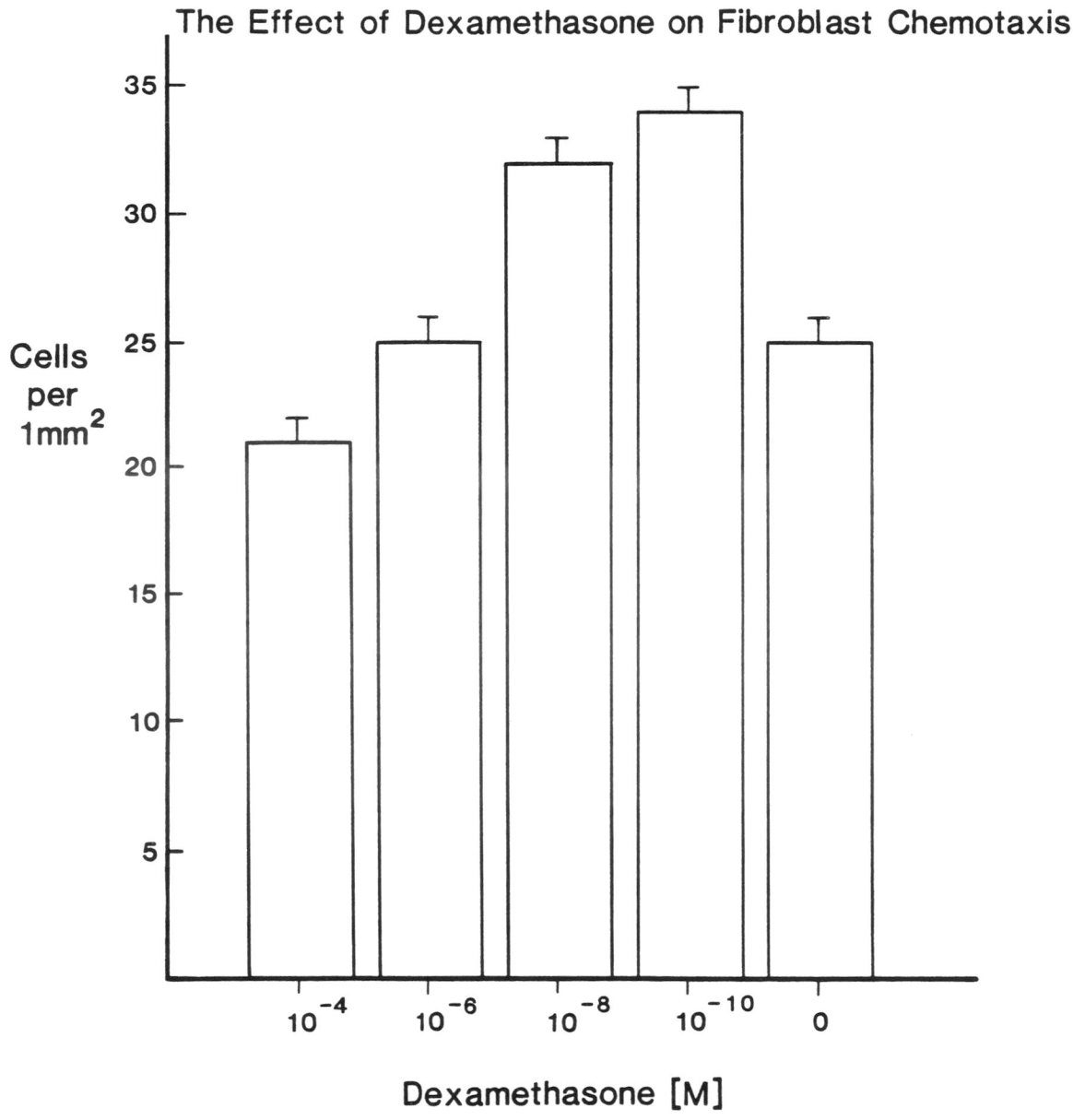
Dexamethasone at a concentration of 1 x 10^{-4}M inhibited fibroblast chemotaxis by 14% (Graph 3). Cell migration in response to 1 x 10^{-6}M was not statistically different from positive controls. This indicated no cellular inhibition or enhancement. Enhancement of fibroblast chemotaxis by dexamethasone was observed at 1 x 10^{-8}M (29%), and 1 x 10^{-10}M (33%). Boyden chambers were also prepared contain-ing Medium 199 in the lower chamber. Upper chambers containing cells treated with 1 x 10^{-10}M dexamethasone failed to stimulate chemotaxis in the absence of conditioned medium. Untreated cells also failed to migrate to dexamethasone, thus ruling out any chemoattraction by inhibitors.

Graph 2 - Fibroblasts were obtained as described in materials and methods and incubated with various concentrations of indomethacin. Cells were then assayed for their ability to migrate towards conditioned medium.

The Effect of Indomethacin on Fibroblast Chemotaxis



Graph 3 - Fibroblasts were obtained as described and subjected to various concentrations of dexamethasone. Chemotaxis assays then determined the effect of dexamethasone on fibroblast chemotaxis towards conditioned medium.


The Effect of Dexamethasone on Fibroblast Chemotaxis
Cells per 1mm2
35
30
25
20
15
10
5
10-4
10-6
10-8
10-10
0
Dexamethasone [M]

DISCUSSION

An initial aspect of this study was to determine and obtain in high yield an active chemoattractant for fibroblasts. Work prior to this study revealed that fibroblasts migrated to maintenance medium which had been exposed to confluent layers of fibroblasts. This medium contained 8% FCS, thus implicating the role of growth factors or hormones associated with FCS in stimulating chemotaxis. With this in mind the possibility of other modified media in stimulating chemotaxis was investigated. A modified medium supplemented with thyroxine, insulin, transferrin and selenium was incubated for three days with confluent layers of fibroblasts, and elicited the highest chemotactic activity. This medium was then utilized for all subsequent assays. This supports work by Mensing (1983), who found that medium conditioned by human fibroblasts elicited chemotactic activity. Thus, the release of chemotactic factors into their medium may serve to regulate such cell specific activities as adherence, migration, replication, and maintenance of the extracellular matrix. Many of the chemotactic factors contained in conditioned medium include: fibronectin (Albini, 1983), collagen (Postlethwaite *et al.*, 1979), and/or their fragments as well as a nonalbumin 60 Kd protein yet to be identified (Mensing *et al.*, 1983)

Electrophoresis of CM revealed many proteins which were not present in MM. This lead to the conclusion that the chemotactic activity of CM resided in the presence of these

proteins. A large majority of the chemotactic activity of CM is due to the secretion of fibronectin, or its fragments, by fibroblasts. Therefore, comparisons were made between co-migrating proteins in samples of CM and fibronectin. The cell attachment segment of fibronectin is located within the central region of both the alpha and beta chains of fibronectin (Sekiguchi and Hakomori, 1983; Ingham et al., 1984; Hiyashi and Yamada, 1984; Sekiguchi et al., 1985). The chemotactic activity associated with this region is due to a chemotactic sequence localized within the COOH terminal end of the cell attachment region (Albini et al., 1983). Fragmentation of the fibronectin chains has determined that this chemotactic region is located 90 Kd from the COOH terminal end of fibronectin. A trypsin (Hiyashi and Yamada, 1983; Sekiguchi et al., 1983), elastase (Ingham et al., 1984) and cathepsin D (Albini et al., 1983) digest of the fibronectin molecule releases the cell attachment region as a 140-155 Kd polypeptide fragment. A subsequent plasmin digest of this fragment releases a 90 Kd polypeptide which retains chemotactic activity (Albini et al., 1983). A SDS electrophoresis of CM and fibronectin revealed the presence of both the 150 and 90 Kd polypeptides. Both samples also contained a 60 Kd protein which may represent the terminal heparin/fibrin binding regions of fibronectin (Hiyashi and Yamada, 1983). A 28 Kd protein was also contained within CM and may actually be a fibronectin fragment with heparin binding ability. This is based upon the generation of a heparin binding 30 Kd peptide by thermolysin fragmentation

of the cell attachment region. Therefore, the 150 Kd band within CM may represent the cell attachment region of fibronectin, with the 90 Kd band representing a subfragment of the cell attachment region. The chemotactic activity within CM may reside in the presence of chemotactic amino acid sequences within fibronectin and its fragments. Elimination of fibronectin from conditioned medium results in a 50-60% reduction in its chemotactic activity (Mensing et al., 1983). Remaining activity is accounted for by the presence of a protein in the size range of albumin (Mensing et al., 1983). Therefore, the 60 Kd protein observed in the CM may be either a 60 Kd fibronectin fragment containing the chemotactic sequence or an unidentified chemotactic protein. Further work involving antibodies raised against fibronectin fragments should resolve this discrepancy.

Damage to tissue results in the release of a wide variety of inflammatory mediators. These include AA metabolites, as well as C5a, PDGF, lymphokines, collagen,and tropoelastin/elastin with their fragments. These compounds serve as chemoattractants in eliciting cellular influx into the damaged tissue sites. Of primary importance in the acute inflammatory response is the metabolism of AA, which generates a wide variety of compounds that mediate the inflammatory response. Of importance are the LTs and Hetes, which elicit cellular influx into the wound site. Inflammatory cells migrating to the wound site also release chemoattractants, thereby enhancing the signal for cellular influx. Thus, chemoattractants serve as cell to cell

signals in eliciting cell migration, and ultimate tissue reconstruction, in response to tissue damage. Arachidonic acid metabolism not only generates chemoattractants but serves as the principal biochemical mechanism in chemotaxis. The Ca^{+2} influx associated with this pathway may activate a wide variety of kinases, or serve to initiate the polymerization of microtubules and microfilaments. Therefore this study attempted to determine how inhibition of specific branchpoints in AA metabolism would affect the chemotactic response of fibroblasts.

Indomethacin, a nonsteroidal anti-inflammatory agent and dexamethasone, a steroidal glucocorticoid, were utilized as inhibitors of AA metabolism. Indomethacin (10^{-6}M) is a irreversible competitive inhibitor of cyclo-oxygenase, thus inhibiting the formation of PGs, PGI, and TxA_2 from AA. A reduction in prostaglandin levels would result in a decrease in the redness, swelling and pain at the wound site. The potency of this drug in inhibiting cyclo-oxygenase resides in the presence of the N-benzyl and -methyl acetic acid side chains which are attached to the indole ring of indomethacin (Lombardino, 1985). Inhibition of PG synthase by high concentrations of indomethacin (10^{-4}-10^{-6}M) resulted in an enhanced chemotactic response by the fibroblasts (Table 2). This response decreased with lower concentrations of indomethacin, and cells treated with 10^{-10}M indomethacin were not significantly different from controls. A chemotactic enhancement due to inhibition of prostaglandin production has been reported previously in

Table 2 - A Summary of the effects of Indomethacin and Dexamethasone on Fibroblast Chemotaxis.

Fibroblast Chemotaxis in Response to Anti-inflammatory Agents

	Indomethacin (n = 7)		Dexamethasone (n = 6)	
Concentration	Cell per 1mm	% (−) Inhibition / (−) Enhancement	Cells per 1mm	% (−) Inhibition / (+) Enhancement
10^{-4}	21.79 ± 1.07	+91 %	21.42 ± 0.76	−14%
10^{-6}	20.43 ± 1.07	+79%	24.92 ± 0.84	−0.3%
10^{-7}	14.57 ± 0.64	+27%		
10^{-8}	13.86 ± 0.77	+21%	32.33 ± 0.82	+29%
10^{-10}	11.64 ± 0.78	+1.8%	33.17 ± 1.12	+33%

neutrophils (Higgs et al., 1980; Goetzl, 1980; Malmsten et al., 1980; Palmblad et al., 1980). This chemotactic enhancement may be the result of a substrate diversion by AA. This involves the inhibition of PG synthase, resulting in an excess concentration of free AA. This free AA may then be acted upon by 5-lipoxygenase, thereby, increasing the production of LTB_4 (Salmon et al., 1983). Leukotriene B_4 is a potent chemoattractant for fibroblasts and its production could result in an enhancement of chemotaxis. Enhancement of chemotaxis was also observed with cells treated with 10^{-8}-10^{-10}M dexamethasone (Table 2). This may be due to the ability of low concentrations of glucocorticoids to stimulate the production of fibronectin (Marceau et al., 1980, Oliver et al., 1983; Lien et al., 1984). Oliver and co-workers have proposed that two mechanisms govern fibronectin synthesis. One is responsible for basal rates of fibronectin synthesis, which is glucocorticoid independent; and the other is responsible for induced rates of fibronectin synthesis, which is regulated by glucocorticoid receptors. Dexamethasone treatment of fibroblasts results in a two fold increase in the rate of fibronectin biosynthesis in normal cells, and a ten fold increase in fibrosarcoma cells (Oliver et al., 1983). Treatment of fibroblasts with hydrocortisone results in a 13-19% increase in fibronectin levels, with a more dramatic effect in the accumulation of fibronectin in the medium (Lien et al., 1984). Thus, the enhancement observed with 10^{-8} to 10^{-10}M dexamethasone treated cells may be due to an

enhanced production or presence of fibronectin within the upper well of the Boyden chamber. This could then result in an up-regulation of fibronectin receptors. Detection of a higher chemotactic gradient towards CM would then result in chemotaxis. An inhibition of chemotaxis was observed with 10^{-4}M dexamethasone treated cells. This may be attributed to the induction of a chemotactic regulatory protein known as lipocortin (Blackwell et al., 1980; Hirata et al., 1980). Lipocortin is found within a wide variety of cells and phosphorylation of this protein results in the loss in its ability to inhibit PA_2 and chemotaxis (Hirata et al., 1980). Chemoattractant binding results in a Ca^{+2} influx which activates PA_2 and either a Ca^{+2} dependant kinase or c-AMP dependant kinase. These kinases would then phosphorylate lipocortin and render it inactive. Treatment of cells with glucocorticoids results in a rapid release of existing pools of lipocortin from cells, followed by a slower release of inhibitor due to subsequent protein synthesis and release (Walker et a1.,1986). Following a two hour treatment with glucocorticoids, there is a six fold increase in lipocortin-specific mRNA's (Walker et al., 1986). Therefore, the induction of lipocortin by 10^{-4}M dexamethasone results in the inhibition of PA_2 which prevents AA metabolism and fibroblast chemotaxis.

Glucocorticoids have been classically described as having the ability to enhance gluconeogenesis, inhibit glucose and amino acid uptake, and catecholamine O-methyl transferase. They are further implicated in the stress

response involving a synergistic enhancement and prolongation of hyperglycemia, initially induced by epinephrine. Current evidence points to a more direct role for glucocorticoids, i.e., terminators of the anti-inflammatory response. This is due to their ability to inhibit AA metabolism, chemotaxis, lymphokine and interferon production, and Fc expression (Munck et al., 1984). Thus, the release of glucocorticoids following an inflammatory response may serve as a terminating signal in this response, thereby protecting the host from excessive cellular influx, hydrolytic enzyme release and prolonged immunological stimuli. Decreases in circulating glucocorticoid levels would then result in a lower concentration of glucocorticoids in peripheral tissues. These lower levels of glucocorticoids would then induce fibronectin synthesis in preparation of connective tissue repair. Therefore, glucocorticoids serve as a signal to end the acute inflammatory response and initiate a reparative phase whereby, fibroblasts reconstruct the connective tissue matrix.

Dexamethasone and indomethacin are termed anti-inflammatory agents due to their ability to inhibit prostaglandin production. However both compounds under certain conditions may stimulate chemotaxis. Excessive or prolonged use may alleviate pain and swelling, but may also enhance cellular influx into the wound site. This chemotactic enhancement would result in excessive cell proliferation, enzyme release, and generation of radicals,

and tissue destruction. Subsequent research should involve the utilization of dual inhibitors that selectively prevent both PG and LT production. Prostaglandin synthase requires a hydroperoxide activator and a heme group for the catalysis of AA to PGH_2 (Kulmacz, 1986). Thus, the synthesis and availability of inhibitors of peroxide incorporation or peroxide scavengers may result in the availability of a nontoxic class of inhibitors of both chemotaxis and AA metabolism. Other inhibitors may involve radical scavengers, or substrate analogues such as the omega 3 fatty acids. Research is also required in the determination of the effects of anti-inflammatory agents on cell proliferation, collagen synthesis, and collagenase/elastase activity.

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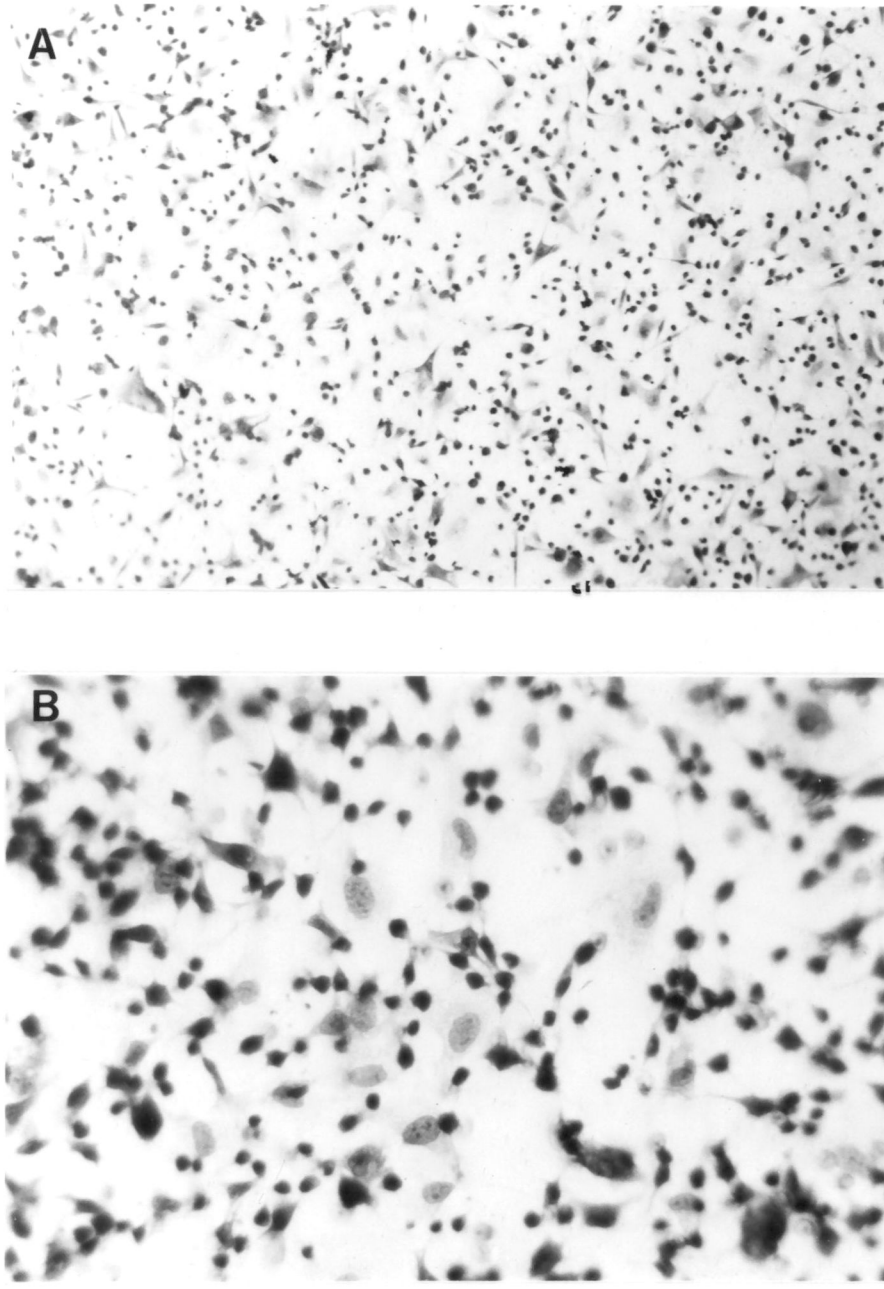
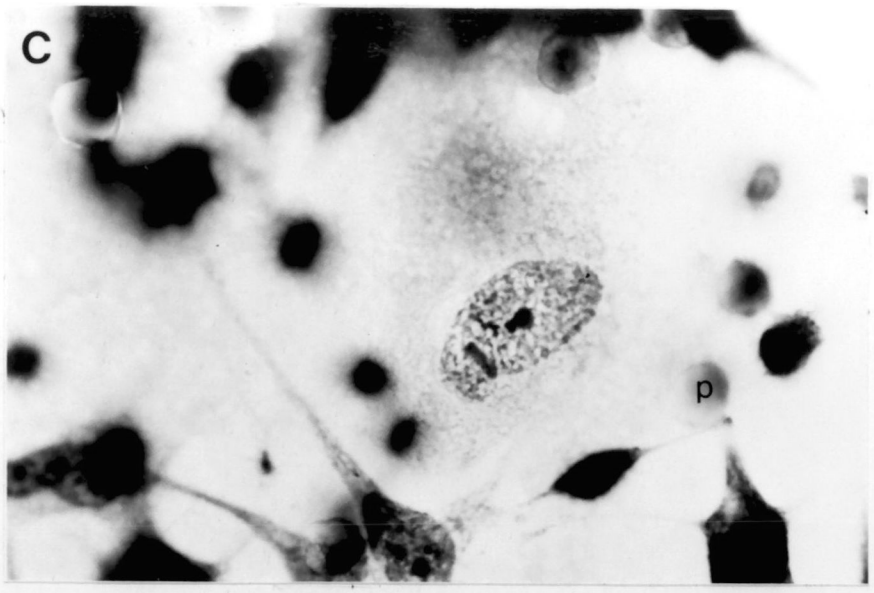
APPENDIX I

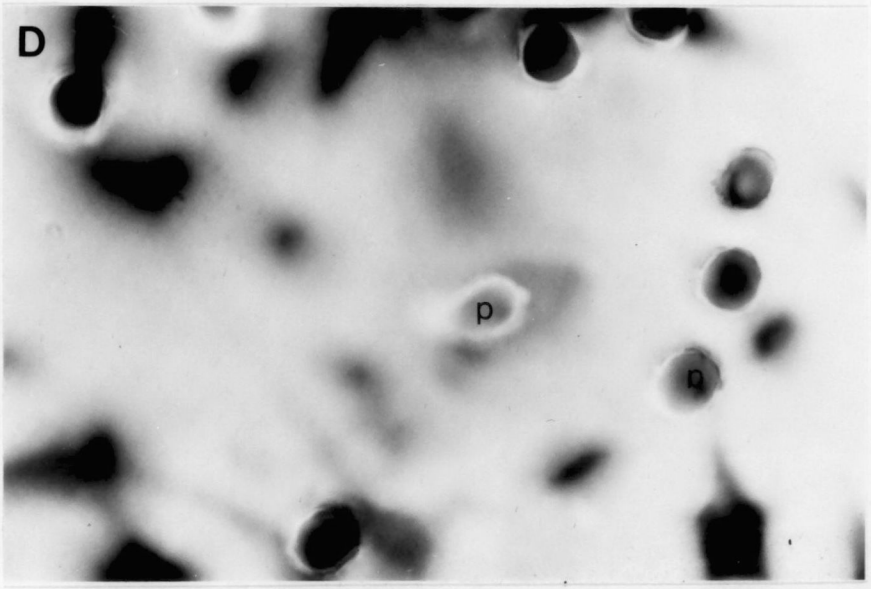
Morphological Studies of Fibroblast Chemotaxis



Light micrographs of both activated and quiescent fibroblasts on a nucleopore filter; A) 100X, B) 400X.

Thru focus series of fibroblast migration in response to a chemoattractant; (C) top of filter, (D) middle of filter, (E) bottom of filter, (p) pore. 1,000X.


C
p


D
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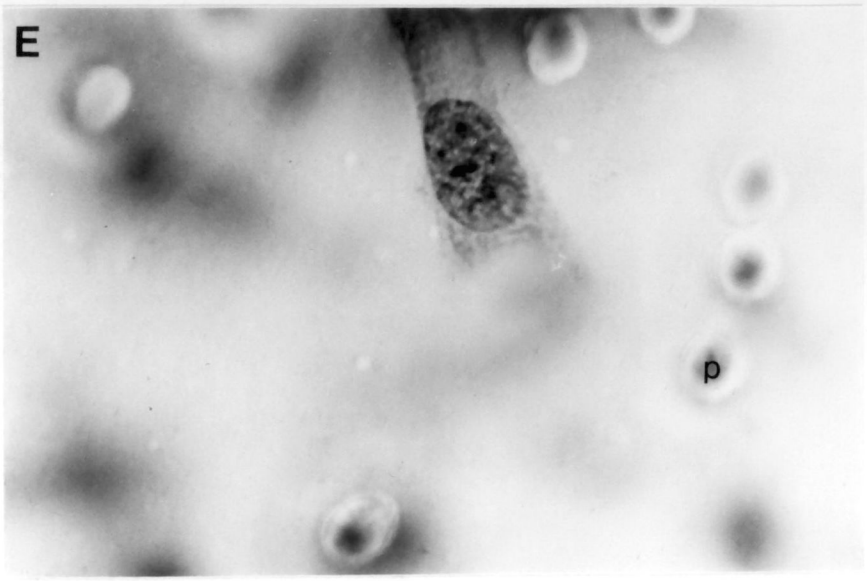
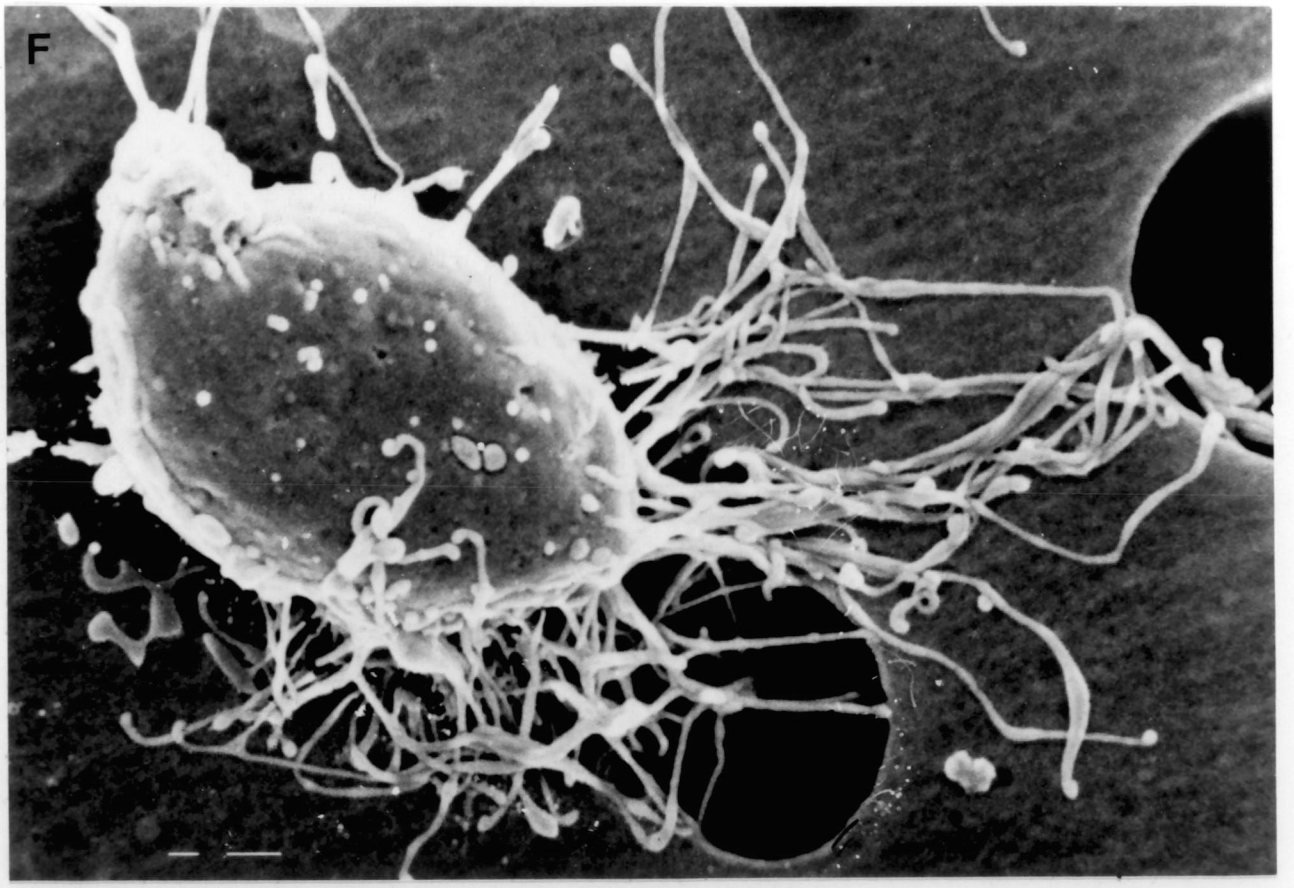
p


E
p

Scanning electron micrograph of; (F) a fibroblast responding to a chemoattractant located beneath the filter, note the filopodia which serve in chemoreception and motility, and (G) a fibroblast oriented on top of the pore in response to a chemoattractant. 5,000X.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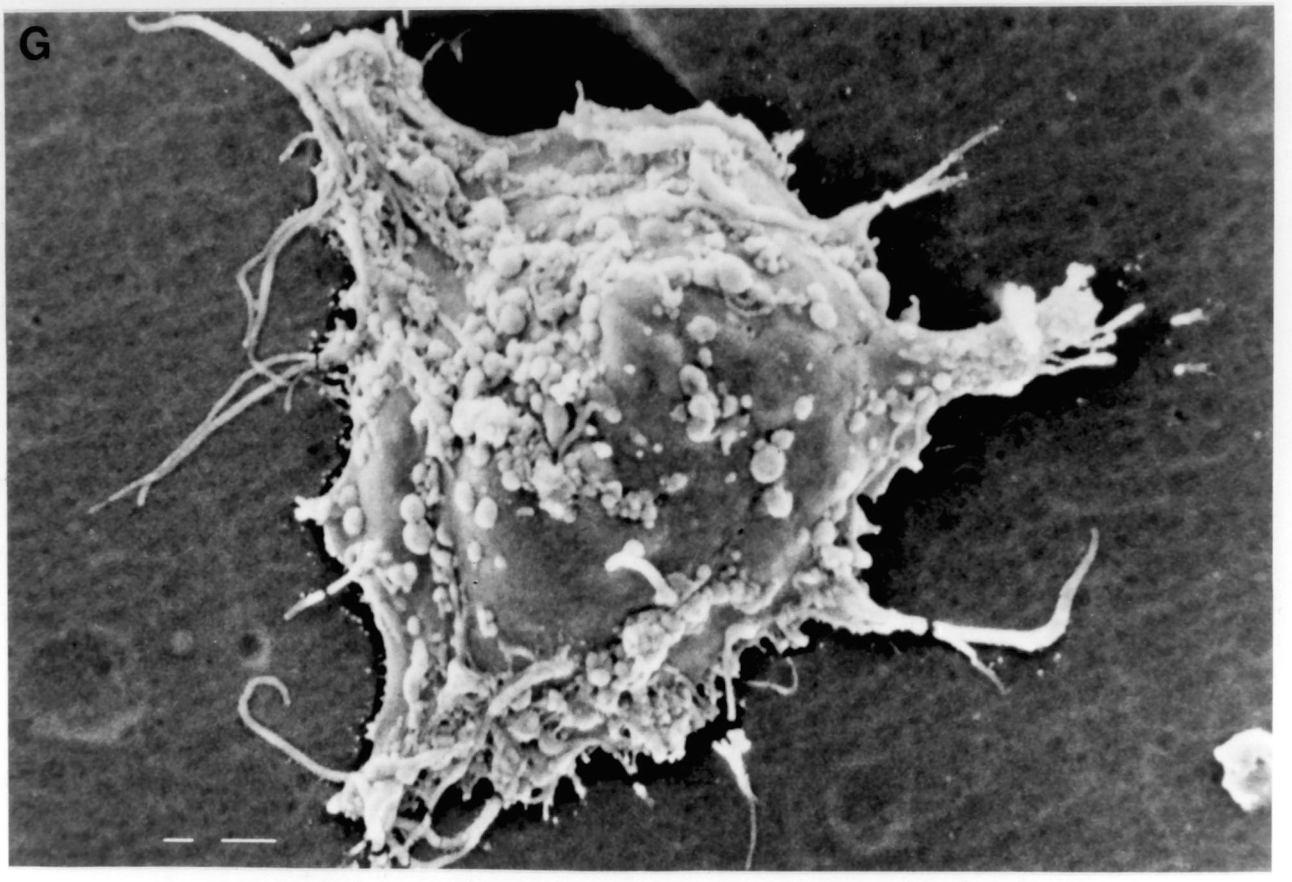
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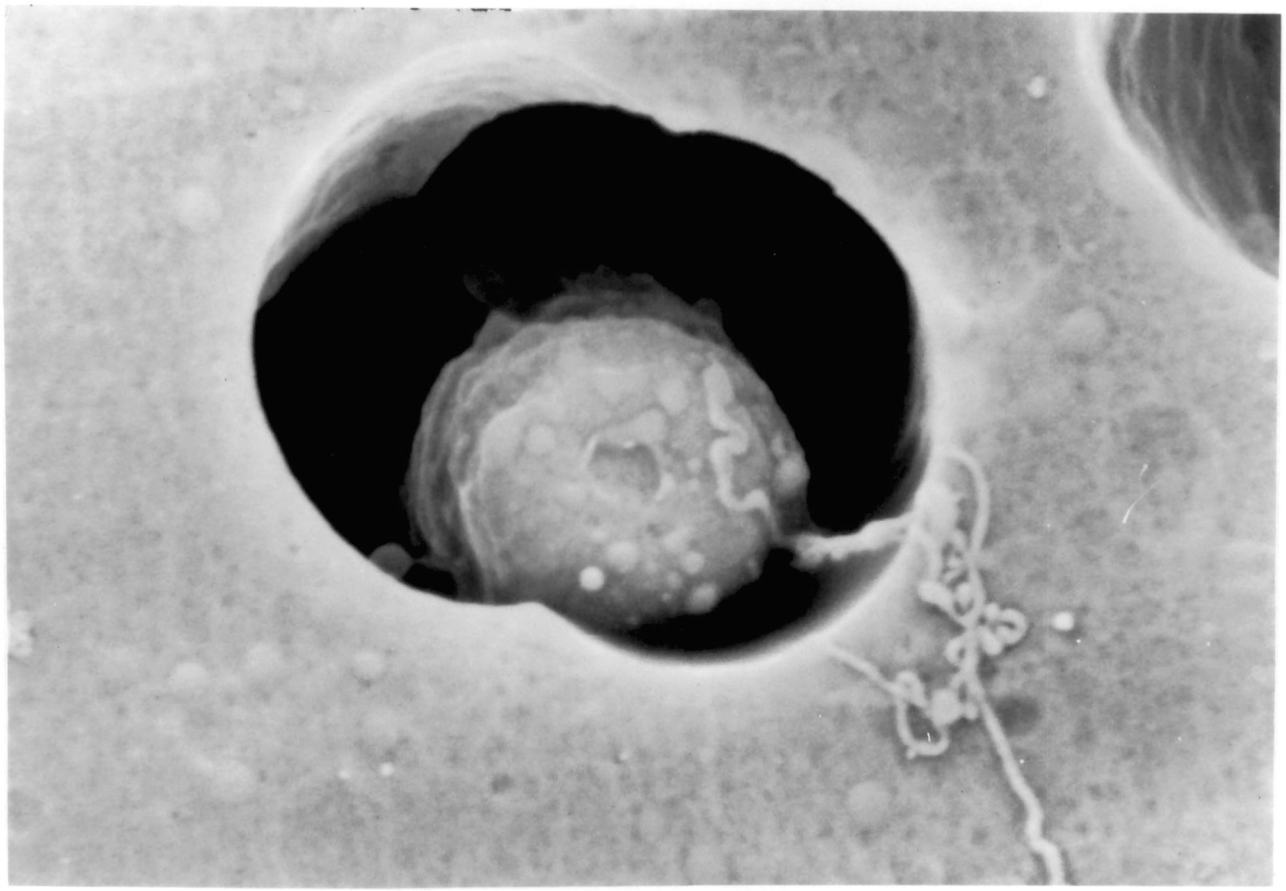

Posterior view of fibroblast within the pore. 10,000X.