MOLECULAR MECHANISM(S) OF THE BIOLOGICAL ACTIVITIES OF CURCUMIN

Thesis

Submitted in partial fulfilment of the requirement for the

Doctor of Philosophy

by

Deepa Thaloor

Under the supervision of

Radha K. Maheshwari, Ph.D.

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI, INDIA

DECEMBER, 1995

Name of Student: Deepa Thaloor

Name of Supervisor: Dr. Radha K. Maheshwari

Professor, Department of Pathology,

Uniformed Services University of the Health Sciences,

Bethesda, MD-20814, USA.

Adjunct Professor, Birla Institute of Technology and Science,

Pilani, Rajasthan-333031, India.

ABSTRACT

Several natural substances have been used as therapeutic agents in traditional Indian medicine. Of these, turmeric, the powdered rhizome of the plant *Curcuma longa Linn.*, has long been used for the treatment of inflammatory diseases. Curcumin (diferuloyl methane), the major pigment of turmeric is its active constituent. Curcumin has been reported to posses several biological properties. Present work is directed towards understanding the mechanism of action of some of its biological properties using both *in vitro* and *in vivo* models.

Angiogenesis inhibitors have been known to suppress tumor growth by restricting neovascularization in animals. We examined the effect of curcumin on angiogenesis in an *in vitro* model of endothelial cell differentiation. Human umbilical vein endothelial cells (HUVEC) rapidly align and form network of tubes when cultured on Matrigel, a basement membrane preparation. Treatment of HUVEC with non-toxic doses of curcumin resulted in a dose-dependent inhibition of tube formation. The extent of inhibition was greater in

the presence of 2% serum as compared to 10% serum. Time course studies demonstrated greater degree of inhibition when cells were pre-treated with curcumin, as compared to treatment at the time of plating on Matrigel, or post-tube treatment. To elucidate the mode of action of curcumin, its effect on proteinases was studied since metalloproteinases have been implicated in angiogenesis. Zymograms of curcumin-treated culture supernatant indicated regulation in the gelatinolytic activities of 53-kD and 72-kD proteinases which were characterized as metalloproteinases. Studies on the RNA transcripts of 72-kD and 92-kD metalloproteinases showed a dose-dependent inhibition, with greater regulation of 72-kD proteinase than 92-kD proteinases. These data suggest that the regulation of the expression of metalloproteinases by curcumin may be responsible for its anti-angiogenic activity.

The effect of curcumin on wound healing was studied in rats, guinea pigs, and rabbits using punch wound models. Healing of wounds is a complex series of biological events that involves the interaction of different cell types, growth factors, cytokines, and extracellular matrix molecules. Oral administration of curcumin resulted in faster and enhanced rate of healing compared to untreated control, as evidenced by increased migration of dermal fibroblasts into the wound bed and complete re-epithelialization by histological studies. Extracellular matrix (ECM) proteins like laminin, fibronectin and collagen which are required for cell adhesion, cell migration and tensile strength were observed to increase in curcumin adminstered animals as compared to untreated animals. We have demonstrated greater induction of growth factors like TGF-β and EGF in curcumin-treated animals as compared to untreated animals by immunohistochemical,

in-situ and RT-PCR analysis. The data suggest that curcumin could be enhancing wound healing by induction of ECM proteins and growth factors which are important in wound healing.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

CERTIFICATE

This is to certify that the thesis entitled **"Molecular Mechanism(s) of the Biological Activities of Curcumin"**, and submitted by Deepa Thaloor, ID No. 90PHXF806 for the award of Ph.D Degree of the institute, embodies orginal work done by her under my supervision.

Dr. Radha K. Maheshwari,
Professor, Department of Pathology,
Uniformed Services University of the Health Science,
Bethesda, Maryland, USA
Adjunct Professor, Birla Institute of Technology & Science,
Pilani, Rajasthan, India.

ACKNOWLEDGEMENTS

I am deeply indebted to my advisor Dr. Radha K. Maheshwari, for his guidance and support throughout my research work at the Uniformed Services University of the Health Sciences, USA.

I am grateful to Dr.S.Venkateswaran, Director, Dr.B.M.Mitthal and Prof.I.J. Nagarath, Deputy Director's and Dr. L.K.Maheshwari, Dean, RCD for providing me an opportunity to pursue research at the Uniformed Services University of the Health Sciences, USA.

Thanks are due to Dr. Gurmel S. Sidhu, Dr. Prasad V. Paruchuri, and Dr. Anoop K. Singh for their encouragement and support.

I would like to thank all my friends, Fred and Janet from AV, and staff of LRC for their help in my thesis work.

Special thanks are due to my parents and family for their love, encouragement and support in every stage of my studies.

TABLE OF CONTENTS

LIST OF FIGURES

CURCUMIN

The ground, dried rhizome of the plant *Curcuma longa Linn.*, has been widely used for centuries in indigenous medicine for the treatment of a variety of inflammatory conditions and other diseases (Ammon and Wahl, 1991). The powdered rhizome is commonly called turmeric. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) or diferuloyl methane (shown in Fig.1), is the major pigment in turmeric which is responsible for the yellow color of curry.

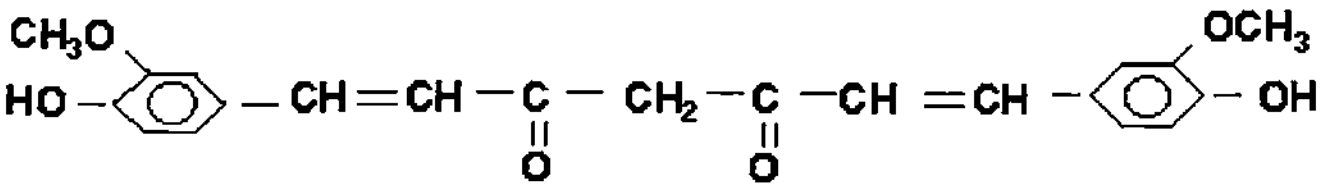


Fig. 1

It is a chrome/orange-yellow, odorless, crystalline compound with mol.wt. 368.37 and mol.formula $C_{21}H_{20}O_6$. It is insoluble in water and ether but soluble in alcohol, glacial acetic acid and dimethyl sulphoxide. It gives a brownish red colour with alkali and a light yellow colour with acids (Merck Index, 1989).

Curcumin has anti-inflammatory (Srimal and Dhawan, 1973; Satoskar et al., 1986) and anti-oxidant activities (Sharma, 1976; Toda et al., 1985). The anti-carcinogenic properties of curcumin in animals has been demonstrated by its inhibition of both tumor initiation induced by benz(α)pyrene and 7, 12 dimethyl benz(α)anthracene (Huang et al., 1992; Azuine and Bhide, 1992) and tumor promotion induced by phorbol esters (Huang et al., 1988) in skin and on carcinogen induced tumorigenesis in the

forestomach, deodenum and colon of mice (Huang et al., 1994). Curcumin has been shown to inhibit TPA-induced progression of epidermal cells through cell cycle (Huang et al., 1992), inhibit smooth muscle cell proliferation (Huang and Jan, 1992), and has inhibitory effect on arachidonic acid-induced inflammation and on arachidonic acid metabolism through inhibition of both the cycloxygenase and lipoxygenase pathways in mouse epidermis (Huang et al., 1994; Huang et al., 1991).

Recently, curcumin has been shown to suppress the proto-oncogenes (Kakkar and Roy, 1994), the transcriptional factor c-jun/AP-1 (Huang and Lee et al., 1991), as well as protein kinase activity (PKC) (Liu et al., 1993; Reddy and Aggarwal, 1994). The inhibitors of PKC may block the phosphorylation induced by TPA-type tumor promoters through inhibition of PKC activity and also block TPA-type tumor promotor induced biological activities and tumor promotion (Nakadate et al., 1986). The suppression of PKC activity by curcumin may cause the inhibition of TPA-induced inflammation and tumor promotion.

Curcumin is a potent scavanger of reactive oxygen species, protects haemoglobin from nitrite-induced oxidation to meth-haemoglobin and inhibits induction of nitric oxide synthetase in activated macrophages (Shalini et al., 1987; Unnikrishnan and Rao, 1992; Brouet and Ohshima, 1995). Some of these activities are also responsible for its ability to protect DNA from free radical-induced damage and to protect hepatocytes against various toxins (Donatus et al., 1990). Recently Dikshit et al., (1995) have demostrated the anti-ischaemic property of curcumin in cat's heart. Besides inhibition of HIV proteases (Sui et al., 1993) and HIV integrase (Mazumder et al., 1995), curcumin has also been shown to be highly effective in inhibiting HIV type-I long terminal repeat

directed gene expression and virus replication (Li et al., 1993) and at present is in clinical trials as a therapeutic agent for AIDS. Thus, curcumin appears to be a multi-faceted drug whose mechanism underlying these diverse effects are not fully understood.

Our laboratory in collaboration with the Central Drug Research Institute (CDRI), Lucknow, India, has been studying the effect of natural compunds on wound healing in animal models. Curcumin is used in Indian traditional medicine as an antiseptic and anti-inflammatory. This kindled our interest in curcumin as a possible wound healer. Studies on cutaneous wounds in animal models showed enhanced healing by curcumin treatment. This report demonstrates the possible molecular mechanism of enhanced wound healing by curcumin. In an attempt to investigate other possible effects of curcumin, we studied the effect of curcumin on angiogenesis in endothelial cells in an *in vitro* model using Matrigel. Angiogenic studies show inhibition of tube formation in a dose-dependent manner by curcumin. Studies on proteinases demonstrated the inhibition of 53-kD, 72-kD and 92-kD metalloproteinases expression by curcumin. The inhibition of angiogenesis may be related to the inhibition of metalloproteinase expression by curcumin.

EFFECT OF CURCUMIN ON ANGIOGENIC DIFFERENTIATION

INTRODUCTION

Angiogenesis is a process which involves sequential events including the migration and proliferation of endothelial cells and subsequent tube formation. This process is fundamental to reproduction, development and repair. Under these conditions, angiogenesis is highly regulated and of short duration. In many pathological states, this regulation is deranged so that the disease itself is driven by persistent, unabated neovasularization. Thus, tumor growth and metastasis are angiogenesis dependent and many non-neoplastic diseases are dominated by uncontrolled angiogenesis. These "angiogenic diseases" include arthritis, psoriasis and many types of ocular neovascularization.

Endothelial cells which give rise to new blood vessels were first grown *in vitro* when human umbilical vein endothelial cells (HUVEC) were cultured (Jaffe et al., 1972). The proliferation of endothelial cells has been found to be a critical event in capillary formation (Ausprunk et al., 1977). This suggested that angiogenic factors should be endothelial mitogens.This idea was further strengthened after the purification of an angiogenic factor, basic fibroblast growth factor (bFGF) which was guided by endothelial DNA synthesis *in vitro* (Shing et al., 1984). However, the next angiogenic factor which was discovered from the conditioned media of a human adenocarcinoma cell line, angiogenin, was not an endothelial mitogen, thereby showing that not all angiogenic factors are endothelial mitogens (Folkman and Klagsbrun, 1987).

Process of angiogenesis

Angiogenesis depends primarily, if not entirely, on endothelial cells, for, it is the endothelial cells that line the lumen of all blood vessels and comprise the entirety of capillaries. Initiation of the process requires that endothelial cells, quiescent in their contact-inhibited state within an existing vessel, are freed from that inhibition. This may occur by mechanical disruption, as in wounding, or by dissolution of the basement membrane, which provides the architectural matrix for continuity of the vasculature. Once released from contact inhibition, endothelial cells leave the previously intact vessel by migrating in the direction of an angiogenic stimulus. Because endothelial cells do not exist singly, but are organized as a tissue, migration must be supported by proliferation, so that, as endothelial cells move distally from the existing vessels, and they preserve continuity by the proximal addition of new endothelial cells. Endothelial proliferation permits extension of the microvascular tubules which develop into loops and then into a functioning circulatory network.

Thus, the "angiogenic cascade", includes:

A. Establishment of vascular discontinuity:

i. Proteolytic enzymes secreted by tumor cells, macrophages or liberated during disruption of basement membranes (Gross et al., 1983) are the most widespread stimulus for angiogenesis. The secretion of proteinases like the metalloproteinases, urokinase and tissue plasminogen activator leads to the disruption of basement membrane.

ii. Local cell destruction or cytolysis brought about by cytotoxic drugs, action of

parasites etc., leads to the release of lysosomal enzymes that modulate basement membrane permeability, thereby mediating vascular discontinuity (Cozzolino et al., 1991).

iii. Endothelial cell retraction that occurs in combination with alterations in the basement membrane architecture. Retraction can be induced by changes in endothelial membrane changes brought about by binding with specific antibodies, lectins or hyperthermia (Fajardo et al., 1988b).

B. Endothelial migration:

i. Chemoattractants: These serve as chemical stimuli thereby inducing the migration of quiescent endothelial cells to migrate from previously contained vessels. There are several chemoattractants discovered such as phorbol myristate acetate (PMA) that induced the migration of cells into collagen gels to form tubes (Montesano and Orci, 1985). An angiogenic factor secreted by hypoxic macrophages has been isolated from wound fluids that are chemoattractants to mesothelial and vascular endothelial cells (Banda et al., 1982).

ii. Cell-matrix adhesion: A critical balance exists between adhesions essential for substrate guidance and adhesions that interfere with cell movement. Adhesion of the cells with the ECM proteins like laminin, fibronectin may either inhibit or enhance cell movement and cell surface reorganization (Grant et al., 1992) which in turn markedly influences the net effect of the interactions between endothelial cells and matrix components.

iii. Divalent cations: Divalent cations are critically important in providing membrane stability. Externally administered chelators such as EDTA, lead to membrane

instability, which in turn triggers cell locomotion. Further, calcium dependent adhesion molecules (cadherins), not only mediate homotypic adhesions between endothelial cells but also mediate interactions between leukocytes and endothelial cells which permit the release of cytokines by the leukocytes and these may tend to modulate endothelial cell movement.

C. Endothelial cell proliferation:

i. Growth factors: Proliferation of endothelial cells is regulated by growth factors. Quiescent endothelial cells achieve significant levels of proliferation during angiogenesis. Fibroblast growth factor (FGF), was one of the first endothelial mitogens discovered from the brain (Gospodarowicz et al., 1978). This was followed by endothelial cell growth factor (ECGS) from hypothalamus (Maciag et al., 1979).

ii. Signal transduction: Signal transduction is initiated once growth factors bind to their receptors on the surface of target endothelial cells. Successful induction of cell proliferation requires interaction of growth factors with their ligands followed by phosphorylation, transport, activation of transcription factors and the resultant signaling which results in the progression of the cell cycle by the endothelial cells.

iii. Cell contact and cell shape: Both cell contact and cell shape can significantly affect the endothelial cell response to growth-stimulating signals. The sensitivity of endothelial cells to tumor necrosis factor alpha (TNFα) depends on their relative rate of proliferation, rapidly dividing cells being more sensitive to TNFα inhibition than more slowly replicating ones. (Van De Viel et al., 1992).

D. Structural reorganization. Two properties of endothelial cells are essential for three-

dimensional reorganization:

i. Endothelial cells have intrinsic polarity manifested by forming a closed loop.

ii. Endothelial cells can form tight junctions with other endothelial cells, thus extending this loop to generate a multicellular array leading to tube formation (Auerbach et al., 1994).

Regulation of angiogenesis

Experiments showing the ability of tumors to induce the growth of new capillaries despite their separation from the vascular bed of the host by millipore filter (Rijhsinghani et al.,1968), demonstrated the release of a diffusible tumor-derived angiogenic factor. The first angiogenic factor was isolated from tumors (Folkman et al., 1971b). Structural studies of isolated angiogenic molecules revealed that angiogenesis is a complex cascade and that an understanding of its regulation would require a detailed knowledge of:

(i) How an angiogenic molecule is exported out of the cells?

(ii) How these molecules are mobilized from extracellular matrix?

(iii) How extracellular matrix itself governs the response of endothelial cells to growth factors?

(iv) How non-endothelial cells like mast cells and macrophages may modify angiogenesis?

(v) How natural inhibitors of endothelial proliferation normally maintain vascular endothelium in a quiescent state?

(vi) How proteases affect tube formation and branching?

(vii) How other systems, such as the endocrine system, may regulate angiogenesis, eg., in the ovarian follicle?

To study the mechanism and regulation of angiogenesis, several models were reported in the literature. These are:

i. The corneal micropocket technique, which permitted linear measurement of individual capillaries as they grow towards a tumor or an angiogenic substance implanted in the cornea (Gimbrone et al., 1974).

ii. The chick embryo chorioallantoic membrane (CAM) was used to detect angiogenic activity of partially purified fractions from tumor extracts (Ausprunk et al., 1974). In this, the test substance is usually adsorbed onto a polymer disc from which it is released slowly.

iii. The subcutaneous rabbit sponge model in which the angiogenic stimulators/ inhibitors, is implanted into polyester discs in rats after creating a lesion (Andrade et al., 1987) and the sponges are gradually infiltrated by blood vessels.

iv. Rat-aortic ring model, wherein thoracic aortas are excised, cultured in collagen gels and treated with the compound under investigation (Diglio et al., 1989).

v. Collagen gels, an *in vitro* model in which the endothelial cells are cultured on the surface of a three-dimensional gel of type I collagen and angiogenic factors studied by their invasion into the underlying collagen matrix.

vi. Matrigel assay: More recently, an *in vitro* differentiation model has been described wherein endothelial cells rapidly and completely formed a capillary like network on reconstituted basement membrane (Kubota et al., 1988; Grant et al., 1989). The basement membrane (Matrigel), contains laminin, collagen IV, nidogen/entactin and

proteoglycan. Matrigel affects a variety of biological activities including cell adhesion, migration, growth and morphogenetic changes during development and differentiation (Carey, 1990). The reconstituted basement membrane protein mixture derived from the Englebreth-Holm-Swarm (EHS) murine sarcoma induced differentiation in a variety of cell types like epithelial cells (Streuli et al., 1991) and bone cells (Vukicevic et al., 1990). Culturing of micro-vascular endothelial cells on Matrigel resulted in the increased expression of factor VIII and uptake of acetylated low density process. Endothelial cells attach to laminin in Matrigel through an RGD (arginine-glycine-aspartic acid) site on the laminin molecule, a second site containing YIGSR (tyrosine - isoleucine - glycine - serine - arginine) induces the cells to form a capillary like network (Grant et al., 1989), and a third biologically active site in laminin, SIKVAV (serine-isoleucine- lysine- valine - alanine - valine), promotes endothelial migration and invasion into Matrigel. Kuzuya and Masafumi, (1994) have shown inhibition in the synthesis of DNA and formation of a network of capillaries when endothelial cells from the bovine aorta are plated on Matrigel. The regression of tubes which occured with time could be prevented by the addition of transforming growth factor-beta (TGFβ) antibodies thereby indicating that TGFβ and other soluble factors from Matrigel may be important for differentiation and remodeling of endothelial cells. When endothelial cells are layered on Matrigel the cells attach and differentiate into capillary like tubes.

Role of proliferation in angiogenesis

During angiogenesis, proliferation of endothelial cells occur at the growing tips of

the capillary tubes. Rogelj et al., (1989) have shown that ECM-associated bFGF induces endothelial cell proliferation. Likewise there are several inhibitors of proliferation eg., TGFβ which inhibit the proliferation of bovine aorta endothelial cells in vitro (Frater-Schroeder et al., 1986). The stimulation in proliferation by FGF was antagonized by TGFβ.

Role of extracellular matrix (ECM) proteins in angiogenesis

Capillary endothelial cells *in vivo* are surrounded by, and rest on basement membranes composed of several extracellular matrix components. The normally quiescent endothelial cells respond to injury and other stimuli by breaching the constraints of the basement membrane and migrating and proliferating toward the stimulus. Thus, throughout the reparative process capillary endothelial cells are in intimate contact with matrix components, thereby suggesting the importance of ECM proteins in directing the dynamic responses of capillary endothelial cells in injury.

As shown in Fig.2, ECM consists of several components such as collagens, glycoproteins (laminin, fibronectin, entactin and nidogen), proteoglycans, and glycosaminoglycans. Of these, collagens form the major constituents of ECM, constituting about 25% of the total proteins. Twelve types of collagen molecules have been identified of which, types I, II, III and IV have been well defined. Different collagen types have different proteolytic requirements for degradation (Mignatti et al., 1993).

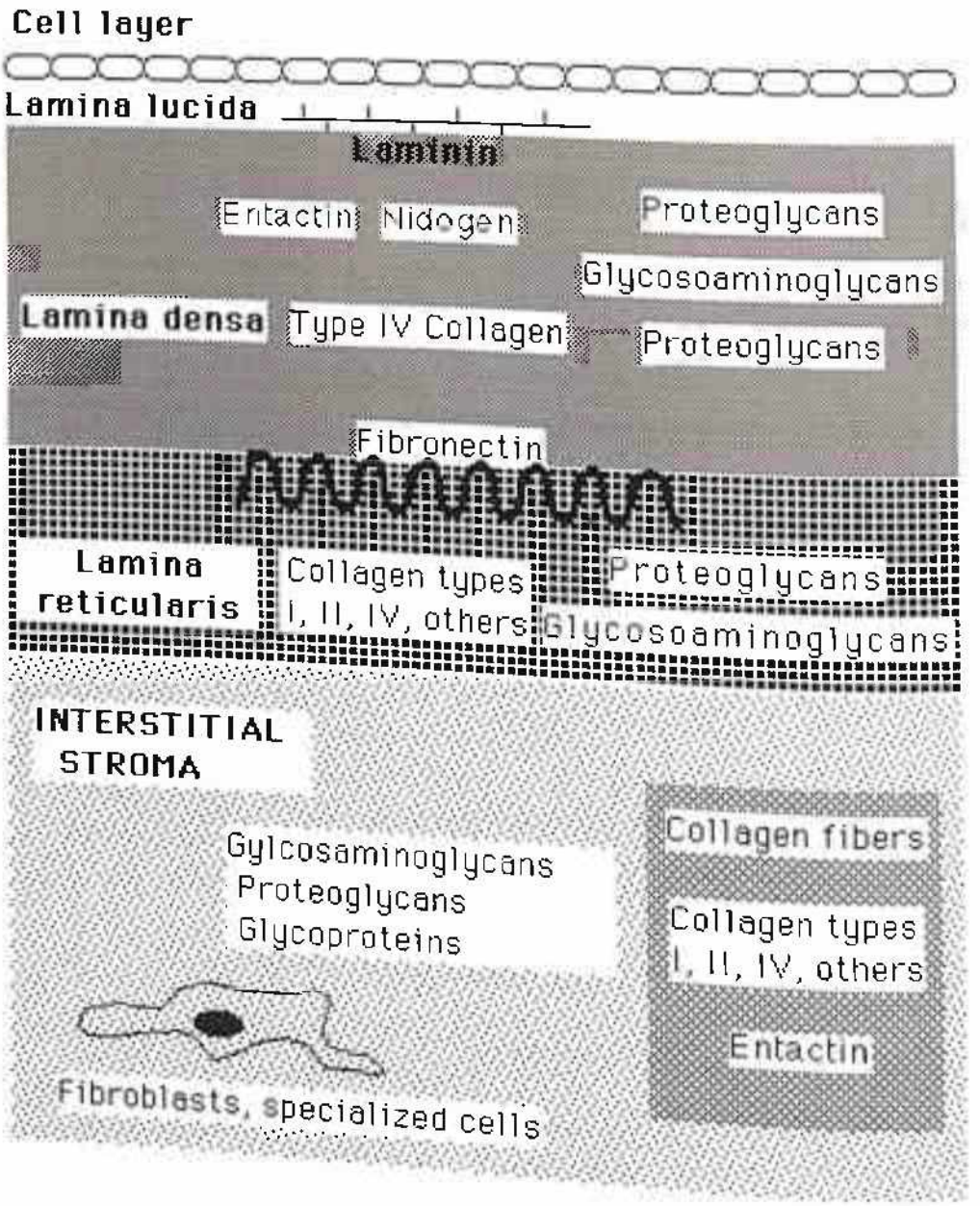
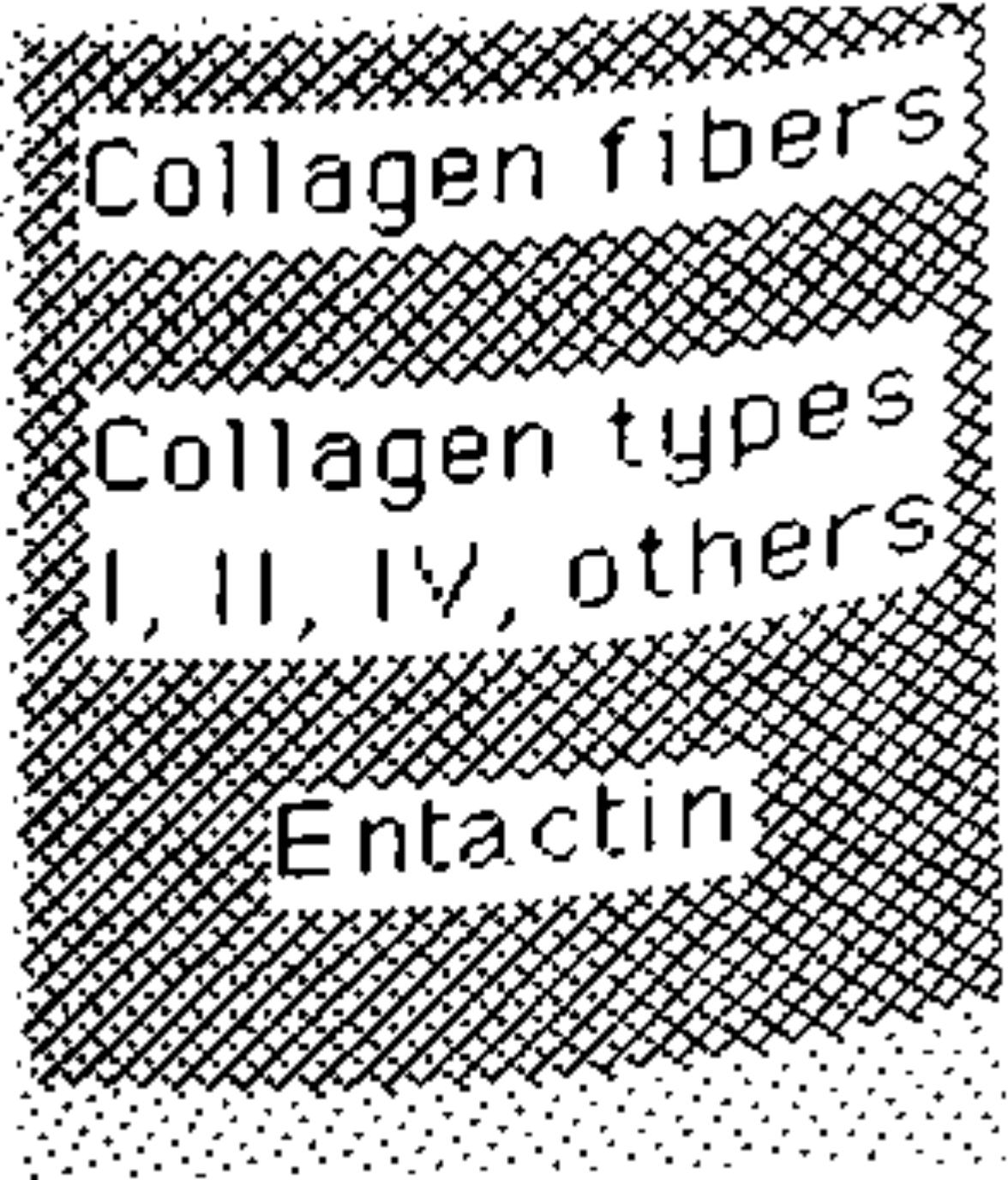


Fig.2: Composition and structure of extracellular matrix


Collagen fibers
Collagen types
I, II, IV, others
Entactin

Of the glycoproteins present in the ECM, the best characterized are the laminin, fibronectin, entactin and nidogen. The fibronectin and laminin contribute to the molecular structure of the ECM by binding ECM components and mediating their attachment to cells. These two glycoproteins also play an important role in determing cell morphology, differentiation and division, and in cell locomotion. The connective tissue and parenchymal cells ensure the integrity of ECM by a continuous process of synthesis and degradation of its components. Under normal conditions the basement membrane is impermeable to large proteins. However, they become permeable to cell movement during tissue remodeling and invasive processes, including inflammation, wound healing and angiogenesis. Defective basement membrane organization or loss may be due to decreased synthesis, abnormal assembly or increased degradation of its components or combination of all three mechanisms. The invasion of cells into the ECM also depends on multiple factors, including the ability of cells to interact with specific ECM components and to migrate actively (Liotta et al., 1983). A number of components of the ECM are considered to act as reservoirs for growth factors, proteolytic enzymes and their inhibitors eg., TFG-β can bind to type IV collagen (Paralkar et al., 1991); matrix metalloproteinases (MMP-2 and MMP-9) bind to laminin and reconstituted basement membrane (Mackay et al., 1993).

Study of capillary endothelial cells *in vitro* (Gospodarowitz et al., 1980) has allowed for a systemic approach in which many variables can be controlled thereby mimicking the *in vivo* condition. The endothelial mitogen, FGF, in the range of 0.1-10ng/ml stimulate endothelial chemotaxis and DNA proliferation as well as angiogenesis

in vivo. In response to FGF, capillary endothelial cells have been shown to invade into three-dimensional collagen matrix as a result of increase in the production of urokinase type plasminogen activator thus demonstrating that the angiogenic response to FGF is a direct effect of growth factor and not secondary to an inflammatory response (Montesano et al., 1986). While collagen has some adhesion promoting activity, laminin has been shown to have potent action on cells-stimulating cell adhesion, growth, differentiation and migration (Grant et al., 1981; Kleinman et al., 1985). The importance of collagen in the alignment of endothelial cells on Matrigel *in vitro* as well as during angiogenesis *in vivo* has demonstrated the requirement of collagen as a prerequisite for tube formation (Haralabopoulos et al., 1994).

In the initial stages of angiogenesis, when the endothelial cells have to form capillary sprouts, the cells have to overcome the restraining mechanical barriers imposed by their basement membrane and surrounding matrix. For this to occur, the proteolytic enzymes localized to the cell surface facilitate this process by degrading the matrix components.

Role of proteinases in angiogenesis

During tumor growth, while malignant cells invade normal adjacent tissues, the neoplasm itself is invaded by "normal" vascular endothelial cells from nearby capillaries or venules. These cells form the tumor's vascular network. Across the newly formed vessel wall, two-way invasive processes occur. On the one hand, immune cells egress from the vessels to infiltrate the neoplasm; on the other hand, tumor cells penetrate

through the capillary lumen into the systemic circulation. Some of these malignant cells arrest in capillary beds at sites distant from the primary tumor. Here they may invade through the vessel walls into the surrounding tissue and form secondary tumors (metastasis).

A common feature of invasive processes is the degradation of extracellular matrix (ECM), basement membranes, basal laminae and interstitial stroma required for invasive cells to migrate into adjacent tissues. For the degradation of the different components of ECM, a complex array of lytic enzymes is necessary. Bovine aortic endothelial cells grown on these structures were shown to degrade all the components of the matrices by the production of different proteinases (Schmitt et al., 1992).

The growth of new blood vessels during angiogenesis is associated with three distinct responses by the micro-vascular endothelium. Capillary endothelial cells, which ultimately form the growing tip of a new capillary sprout, must undergo: a. degradation of the basement membrane surrounding intact capillaries and migrate through extracellular matrix towards the source of the angiogenic stimulus; b. proliferate behind the migrating front; and, c. form the vascular lumen.

The induction and arrest of capillary endothelial cell migration during neovascularization and angiogenesis are likely to require precise regulation of the synthesis and degradation of extracellular matrix. Thus, the induction of synthesis of connective tissue proteinases and the regulation of proteinase activity can each be pivotal in controlling tissue remodeling. During angiogenesis, angiogenic factors like acidic and basic FGF (Gross et al., 1982), and TGF-β (Pepper et al., 1990) have been

shown to induce the synthesis of matrix proteinases by endothelial cells. It has been reported that angiogenin, a potent inducer of neovascularization in the CAM and rabbit cornea, promotes endothelial cells invasion of Matrigel basement membrane by stimulation of cell-associated proteolytic activities (Hu et al., 1994). Recent studies have established the potential for capillary endothelial cells to express a type IV collagenase activity (Kalebic et al., 1983) as well as 5-13 times increased metalloproteinase activity when stimulated by phorbol ester (TPA) or angiogenic factors (Gross et al., 1982). Stimulated rabbit capillary endothelial cells (RBCE) express little collagenase and stromelysin activity either before or after activation by trypsin or an organomercurial agent, despite the synthesis and secretion of high concentrations of the zymogens, proCL and proSL, and their conversion to macromolecules of the size of the active proteinase (Herron et al., 1986). The activities of proteinases secreted by the endothelial cells was only about 10-20% as compared to the activity of proteinases from stimulated synovial fibroblasts. The difference has been contributed to the secretion of large amounts of tissue inhibitor of metalloproteinases (TIMPs) by endothelial cells as compared to fibroblasts, inspite of the comparable levels of synthesis and secretion of these proteinases in both the cells. The proteinase activities are thus masked by endogenous metalloproteinase inhibitors like TIMP. Removal of the endogenous inhibitors by chromotagraphy lead to measurable activity of the proteinases (Murphy et al.,1977).

Because ECM components have unique proteolytic requirements for their degradation, it involves an array of hydrolytic enzymes. The ECM degrading proteinases produced by most invasive cells can be subdivided into three classes:(i)

Metalloproteinases eg.,collagenase (ii) Serine proteinases eg.,plasminogen activator, and (iii) Cystein proteinases eg., cathepsin B.

The metalloproteinases produced by invasive cells include enzymes that can degrade different collagen types, laminin, fibronectin and the protein core of proteoglycans. The metalloproteinases involved in ECM degradation (matrix metalloproteinases) share the following features:

(i) Contain a zinc ion at their active site and are inhibited by chelating agents.

(ii) Show consistent sequence homologies.

(iii) Secreted in a latent form and become activated by partial proteolytic cleavage.

(iv) Inhibited by specific tissue inhibitors of metalloproteinases like TIMPs.

Metalloproteinase are divided into three major subclasses based on their substrate specificity:

NAME(s)	SIZE(KDa)	DEGRADES
I. Interstitial collagense	52 deduced	I, II, III collagen
(Type I collagenase/MMP-I)	52, 57 secreted	
PMN collagense (MMP-8)	75 secreted	I, II, III collagen
II. 72KDa Type IV collagenase	72 secreted	IV, V, VII collagen
(72 KDa gelatinase/ MMP-2)		fibronectin, gelatin

92KDa Type IV collagenase (92KDagelatinase/MMM-9)	78 deduced 92 secreted	IV, V collagen
III. Stromelysin (transin/ MMP-3/proteoglycanase)	53 deduced 57, 60 secreted	proteoglycans, laminin, fibronectin, III, IV,V collagen, gelatin.
Stromelysin -2 (transin 2/ MMP-10)	53 deduced	III, IV, V collagen fibronectin, gelatin
PUMP-1 (MMP-7/ small MMP of uterus).	28 deduced	gelatin, fibronectin

---

The expression of MMP activity is controlled at three levels: a. gene transcription, b. proenzyme activation and c. inhibition by specific tissue inhibitors. In most cell types the MMP genes are not constitutively expressed (Matrisian, 1990). However transcription can be induced by a number of agents like phorbol esters (Chin et al., 1985) and oncogene products (Kerr et al., 1990). Recent studies on the regulation of MMP expression have provided strong indications for the involvement of these in tumor progression. TRE (12-O-tetradecanoyl phorbol-13-acetate-responsive element) or AP-1 (activator protein-1) binding site is present in the promotors of rat, rabbit and human stromelysin genes and in the human interstitial collagenase gene (Curran et al., 1988). These binding sites form heterodimeric complexes with the transcription factors c-fos and c-jun. Induction of c-fos protein by Ha-ras and V-mos oncogenes, tumor promotors

or platelet derived growth factor (PDGF) results in the subsequent induction of collagenase and/or stromelysin gene expression (Schonthal et al., 1988).

The translational products of MMP mRNAs are secreted in the proenzyme form. *In vitro* latent MMPs can be activated by agents like organic mercurials. The fully activated MMPs can be inhibited with specific tissue inhibitors of metalloproteinases (TIMPs). It has been shown that the same cells which produce interstitial collagenase are capable of synthesizing and secreting TIMPs. The net enzyme activity results only when activated collagenase levels exceed inhibitor production (Herron et al., 1986 a).

The involvement of proteinases in the differentiation of cells on Matrigel during angiogenesis has been demonstrated using human omental microvascular endothelial cells (HOME cells). It has been observed that the mRNA of tissue-type plasminogen activator and 72kD type IV collagenase increased while that of plasminogen activator inhibitor-1 (PAI-1) and TIMP-2 was increased initially within the first 4h, then decreased and again increased to greater than 18 fold in the expression of the message in HOME cells grown on Matrigel as compared to cells on collagen (Ito et al., 1995).

Thus, metalloproteinases and their endogenous inhibitors are key components of an enzyme system which is important for a number of fundamental biochemical and cellular processes. Like other enzyme families, the metalloproteinases are a key component of a system of "balanced proteolysis" wherein an equilibrium exists between the amount of active enzyme and its endogenous proteinase inhibitor(s).

Serine proteinases include plasminogen activators (PAs), leukocyte elastase, and cathepsin-G. Plasminogen activators convert plasminogen into active plasmin which

degrade several ECM components including fibronectin and laminin. It does not degrade native collagens but can degrade gelatin. Plasmin also activates metalloproteinases (Eaton et al.,1984).

Cystein proteinases include endo- and exo-peptidases. These are intracellular enzymes found in the cytosol or in the lysosomes. These are active against a wide range of small peptides and large protein substrates. Some are able to activate procollagenase or directly degrade collagen and can digest the protein core of proteoglycans (Bond et al., 1987).

Inhibition of angiogenesis

The search for angiogenesis inhibitors was stimulated by the concept of "anti-angiogenic therapy", which states that a putative inhibitor of blood vessel growth might be therapeutic because it could limit tumor growth, and non-toxic because angiogenesis is normally infrequent (Folkman, 1971). The first angiogenic inhibitors to be identified were naturally occuring, or "endogenous" molecules, such as the angiostatic steroid medroxyprogesterone acetate (Gross et al.,1981) which was later identified to inhibit plasminogen activator (Ashino-Fuse et al., 1989) and also change the subunit structure of laminin (Tokida et al.,1990), thereby altering the basement membrane. The efficiency of the angiogenic inhibitors depends upon their route of administration and upon the type of angiogenesis to which they are targeted. For example, angiostatic steroids are most effective when applied topically for corneal neovascularization (Li et al., 1991), while systemic therapy is only effective against tumors (Sakamoto et al., 1988). An

angiogenesis inhibitor fumagillin derived from Aspergillus fumigatus (Ingber et al., 1990) is a potential inhibitor of angiogenesis and of tumor growth when administered systemically. Inhibitors of angiogenesis, like synthetic angiostatic steroids (U-24067 and U-42129), heparin, suramin, interferon-2alpha (IFN-2α), and retinoic acid have been shown to distinctly effect the plasminogen-dependent proteolytic system in human bovine microvascular endothelial cells (Pepper et al., 1994). Johson et al., (1994) have shown the inhibition of angiogenesis by TIMP in an *in vivo* rat corneal angiogenesis assay. The inhibition by TIMP has been partly attributed to the inhibition in the migration of endothelial cells.

Targets of angiogenesis inhibitors

Strategies of anti-angiogenic therapy depends on the knowledge of the biology and biochemistry of the angiogenic process per se. An anti-angiogenic compound may be able to inhibit angiogenesis by the following mechanisms:

1. Inhibit the export of angiogenic molecule from tumor cells.
2. Neutralize angiogenic molecules in transit.
3. Restict endothelial cell proliferation and/or migration.
4. Block the degradation of the basement membrane induced by proteinases.
5. Neutralize tumor-derived chemotactic factors for macrophages or mast cells.
6. Inhibit angiogenic factors secreted by macrophages and/or mast cells.
7. Inactivate tumor-derived plasminogen activators and proteinases.
8. Restrict basement membrane storage of bFGF and other angiogenic modulators.

9. Prevent the accumulation of fibrin products in the neovascular bed.

10. Inhibit enhanced secretion of bFGF by endothelial cells.

11. Increase the secretion of angiogenic inhibitors secreted from tumor cells.

As mentioned earlier, proteolysis is an important prerequisite for several important physiological events, one of which is angiogenesis, wherein new capillary formation occurs. With this in mind, along with reports of the inhibitory action of curcumin on tumor initiation and tumor promotion kindled our interest to study the effect of curcumin on angiogenesis. To characterize the stimulators or inhibitors or to elucidate the mechanisms of angiogenesis, various *in vitro* models using cultured endothelial cells or explants of aorta have been proposed (Madri and Williams,1983; Nicosia and Otttinetti,1990). To study the effect of curcumin on angiogenesis, we have used the Matrigel model. Using Matrigel, we show that curcumin inhibits angiogenesis which could be a mechanism responsible for inhibition of tumor promotion.

MATERIALS

HUVEC were purchased from Clonetics, Inc., San Diego, CA. Media-199, streptomycin, penicillin, gentamicin, fungizone and 0.05% trypsin-EDTA were obtained from Gibco BRL., Gaithersburg, MD. Endothelial cell growth supplement (ECGS) was purchased from Collaborative Research Inc., Bedford, MA. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc., Logan, UT. Curcumin, dimethyl sulphoxide (DMSO), heparin, L-glutamine, phenyl methyl sulphonyl flouride (PMSF), N-ethyl

malemide (NEM), and dithiothreitol (DTT) were purchased from Sigma Chemical Co., St.Louis, MO. Rabbit anti-factor VIII IgG was obtained from Dako Corp., Carpinteria, CA. Monoclonal anti-human MMP-2 was purchased from Oncogene Science, Cambridge, MA. FITC-labeled anti-rabbit IgG was purchased from Organon Teknika, Durham, NC, while goat anti-mouse IgG, conjugated to alkaline phosphatase was obtained from Bio-Rad, Hercules, CA.The cDNA for 67-kD and 92-kD metalloproteinases was kindly provided by Dr. William Schnaper, Northwestern Medical School, Chicago, IL. Matrigel was a generous gift from Dr. Hynda Kleinman, NIDR, NIH., Bethesda, MD.

METHODS

Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

Cultures of HUVEC were maintained in media-199, supplemented with 2-10% FBS, 100μg/ml of ECGS, 100U penicillin, streptomycin and fungizone, 50μg/ml gentamicin and 50U/ml Heparin in 5% CO_2 and 95% O_2 at 37°C. All experiments were carried out between passages 3-7. Cells were checked periodically for the expression of Factor VIII by immunofluorescence.

Immunofluorescence detection of factor VIII expression

Sterilized coverslips were placed in 50mm culture dishes and $5X10^5$ HUVEC (P_1-P_5) were plated in media containing 10%FBS O/N. Cells were washed with cold PBS and fixed in PBS containing 4% formaldehyde and 5% sucrose for 15min followed by

5min in Triton X-100. The cells were then washed for 15min, three times in PBS. Fixed cells were blocked with 5% bovine albumin in PBS for 15min. The coverslips were incubated in anti-factor VIII rabbit IgG (Dako Corp., Carpenteria, CA) for 30min. The cells were then washed three times for 15min each with PBS and incubated with FITC-conjugated anti-rabbit secondary antibody for 30min. The cells were washed in PBS three times for 15min each, mounted in fluromont and viewed under a Zeiss microscope.

Cell proliferation assay

HUVEC ($1X10^5$) were seeded in 24 well tissue culture treated (Costar, Amherst, MA) plates in 0.5 ml, medium 199 containing either 2 or 10% FBS. After 12h, the media was replaced with fresh media containing 1μCi/ml ^{3}H-thymidine (NEN, Boston, MA) and treated with various concentrations of curcumin (0.25μM-100μM) in triplicate. The experiments were terminated after 3, 6, 12, 24 and 32h of treatment. The cells were washed with cold PBS and treated with 20% TCA for 15min. TCA was then washed away and the cells lysed with 0.1N NaOH containing 0.1%SDS. The lysate was transferred to scintillation vials and counted on Beckmann liquid scintillation counter. DNA synthesis was assayed by measuring the radioactivity incorporated into TCA-insoluble material.

Cell Viability Assay

To determine the effect of curcumin on cell viability, HUVEC ($3x10^4$cells/well),

were cultured in 96 well plates (Costar, Amherst, MA.) in either 2% or 10%FBS containing media. Cell viability was measured using the cell proliferation kit (Boehringer Mannheim, Indianapolis, IN), which assays cell growth by cellular conversion of tetrazolium salt into blue formazan crystals. Cells were treated with varying doses of curcumin (1-25μM) for 6h. Following treatment, 10μl of MTT reagent was added to each well and after 4h, 100μl of a solution containing 10% SDS in 10mM HCl was added, and plates shaken to dissolve the reduced MTT crystals. The viability of the cells was measured by comparing optical density of the treated cells with untreated control in an ELISA reader scanned with a 570nm filter.

Angiogenesis Assay

Twenty four-well culture plates were coated with 0.3ml of Matrigel (14-20mg protein/ml), which was then allowed to solidify at 37^{0}C for 1h. Medium-199 (250μl) containing 2 or 10% FBS was added to the Matrigel-coated wells. HUVEC were then trypsinised using trypsin-EDTA (Gibco BRL., Gaithersburg, MD), washed, suspended in appropriate media and added to Matrigel coated wells (40,000 cells/well in 250μl media). Stock solutions of curcumin (1-10mM) were prepared in DMSO. Various concentrations of curcumin were added to the culture from these stock solutions such that, DMSO had no effect on endothelial cell differentiation. The cells were incubated for 6h at 37^{0}C, in a 5% CO_2 humidified atmosphere, the culture supernatant was aspirated and the cells were fixed with Diff-Quick Solution II (Baxter, McGraw Park, IL). The area covered by the tube network was quantitated using an optical imaging technique,

wherein, the pictures of the tubes were scanned in Adobe Photoshop, and quantitated in NIH Image program. Each dose of control or test compound was assayed in triplicate. In addition, all experiments were performed at least three times and the results are expressed as the mean and the standard error of the mean, from at least three such experiments.

For pre-treatment studies, 2% FBS containing media was added to the cells and incubated overnight. The cells were then treated with curcumin (1-25μM) for 3h. Following treatment, curcumin was washed off, the cells were trypsinized and 40,000 cells /well of each treatment was plated in triplicate on Matrigel coated plates. Tube formation was observed after 6h. Post-treatment studies were carried out by treating tubes formed on Matrigel for 6h with curcumin (1-25μM). Tubes were observed every 2h, and after 12h the tubes were fixed and stained.

Zymogram Analysis

SDS-substrate gels were prepared by a modification of standard SDS-PAGE electrophoresis (Birkedal-Hansen & Taylor, 1982). Type I gelatin was added to the standard acrylamide polymerization mixture at a final concentration of 1mg/ml. The culture media from cells cultivated on plastic were estimated for protein content using protein estimation kit (Pierce, Rockford, IL). Equal protein from each sample was mixed (3:1) with sample buffer (10% SDS, 4% glycerol, 0.25M Tris-HCl pH 6.8 and 0.1% bromo phenol blue) and loaded into the wells of a 4% acrylamide stacking gel. Gels were run at 15mA/h while stacking, and 20mA/h during the resolving phase at 4°C. After

electrophoresis, the gels were soaked in 2.5% Triton X-100 with gentle shaking for 30min at ambient temperature with one change of detergent solution. The gels were rinsed with water for 20min and incubated overnight at 37^{0}C, in substrate buffer (50mM Tris-Hcl buffer, pH 8, 5mM $CaCl_2$ and 0.02% Tween 20). After incubation the gels were stained for 1h in 0.5% Coomassie Blue R-250 in acetic acid:methanol:water (1:3:6), destained in acetic acid:methanol:water (1:4:6). After intensive destaining, proteolytic areas appeared as clear bands against a blue background.The molecular weights of the proteolytic bands were determined in relation to the reference marker proteins, which were simultaneously loaded in the gel. The molecular weight markers were: Myosin 205,000; β-galactosidase 121,000; Bovine serum albumin 86,000; Ovalbumin 50,500; Carbonic anhydrase 33,600; Soybean trypsin inhibitor 27,800; Lysozyme 19,400 and Aprotinin 7,400 (Bio-Rad, Hercules, CA).

Characterization of the Proteinases

For the characterization of proteinases, proteins were separated on SDS-PAGE gels and the gels were incubated for 18h in substrate buffer containing either 10mM EDTA, 1mM PMSF, 5mM NEM or 5mM DTT. Characterization of proteinases was based on comparison of the gelatinolytic activity of proteinases in the presence and absence of inhibitors.

Immunoblotting

HUVEC were plated in 6-well tissue culture plates in medium-199 containing 2%

serum. The cells were conditioned overnight in 2% FBS containing media and treated with various concentrations of curcumin for 6h. Equal amounts of protein was denatured and resolved by electrophoresis on 10% SDS-PAGE gels using standard techniques (Laemmli, 1970) and transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) at 4^0C for 4h. Non-specific binding was prevented by blocking in 5% non-fat dried milk for 4h in TBST buffer (100mM Tris-HCl, pH 7.5, 0.9%w/v NaCl and 0.1%v/v Tween 20). Blots were washed three times for 15min each at room temperature with TBST and incubated individually with anti-MMP-2 monoclonal antibody (Oncogene Science, Cambridge, MA) at 4^0C for 12h. Blots were again washed in TBST and immersed in alkaline phosphatase-conjugated goat-anti-mouse IgG (Bio-Rad, Hercules, CA), followed by washing with TBST. Immunoreactivity was visualized by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (Promega, Madison, WI).

Northern Analysis

HUVEC (8X 10^6) at passage 5 or 6 were plated in large 150cm^2 tissue culture flasks. After 12h the medium was replaced with fresh media-199 containing 2% FBS. Cells were treated with curcumin (1- 25μM) for 3h, and washed with cold PBS. They were lysed in TRIzol (Gibco BRL., Gaithersburg, MD) to isolate RNA as specified by the manufacturer. Further purification of the RNA was done by precipitation with 0.3M sodium acetate and ethanol. RNA was quantitated by measuring its absorbance at 260nm in a Beckmann DU640 spectrophotometer. Equal amounts of RNA (10μg) was

denatured in 6x loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% w/v glycerol, 1.2% SDS and 60mM sodium phosphate pH 6.8) at 75°C for 5min. RNA was electrophoresed on a 1% agarose gel at 3-7V/cm in 10mM sodium phosphate buffer, pH 6.8 with continuous circulation of buffer. The separated RNA was stained with ethidium bromide and photographed under UV light. The RNA was then transferred to Nytran membrane from (Schleicher & Schuell, Keene, NH) by capillary method in 20XSSPE. Transferred RNA was crosslinked by UV light for 2min and hybridized with cDNA probes of 67-kD and 92-kD metalloproteinases. The cDNA was labeled with α^{32}P-dCTP (Amersham, Arlington Heights, IL) using the Oligolabelling kit (Promega, Madison, WI) and purified using nick columns (Pharmacia, Piscataway, NJ). The labeled probe ($1X10^6$cpm/ml) was added to the blot which had been previously pre-hybridized for 8h at 42°C with Hybrisol, a hybridization solution (Oncor, Gaithersburg, MD) and incubated at 42°C for 16h with the labeled probe. The blot was washed with 1xSSPE containing 0.1%SDS, three times at room temperature and once with 0.1XSSPE containing 0.1%SDS for 30min at 50°C. The radioactivity in the blot was measured in Betagen 603 blot analyzer (Betagen, Waltham, MA) for 6h and bands quantitated. The blot was then exposed to X-ray film for 12h and then developed in a Kodak X-OMAT20 autoprocessor.

RESULTS

Expression of factor VIII by HUVEC

HUVEC used in all our experiments expressed factor VIII as demonstrated by

immunofluorescence studies indicating that the cells are still endothelial in nature (Fig.3)

Effect of curcumin on proliferation

Endothelial cells plated on 24 well costar plates in media containing 10% serum were treated in triplicate with curcumin (1-25μM) dissolved in DMSO. Thymidine incorporation assays carried out after 6h curcumin treatment showed inhibition in proliferation in a dose dependent manner (Fig.4A). DMSO concentrations used, showed no inhibitory effect on cell proliferation.

To determine the effect of curcumin on the proliferation of endothelial cells in presence of 2% serum, HUVEC were plated on 24 well plates and incubated in media containing 2% FBS overnight. Media was replaced by fresh media containing 2% serum and ^{3}H-thymidine. Curcumin (1-25μM) was added to the cells in triplicate and cell proliferation was assayed after 6h. Proliferation studies demonstrated an inhibition in proliferation by curcumin in a dose dependent manner (Fig.4B). DMSO, concentrations used did not have any effect on proliferation. The degree of inhibition was greater in presence of 2% serum as compared to the effect in presence of 10% serum.

Effect of curcumin on viability

To ensure that the curcumin doses used in our studies were non-toxic to HUVEC, the effect of curcumin on cell viability was studied using MTT assay. As shown in Fig.5A, curcumin did not affect the viability of HUVEC in the presence of medium supplemented with 10% FBS. Although the viability of HUVEC in 2% FBS containing medium was

unaffected when treated for 6h with low doses of curcumin (Fig.5B), higher doses led to a partial inhibition in viability. The effective concentration required to inhibit viability in 10% serum containing medium however, was higher than the concentration required to inhibit the viability in the presence of 2% serum.

Fig.3 Expression of Factor VIII by HUVEC: HUVEC were periodically checked for the expression of factor VIII. Cells were plated on coverslips overnight. The cells were fixed in formaldehyde and sucrose, and treated with/without monoconal anti-factor VIII antibody for 30min at 37°C. The cells were washed in PBS and incubated for 30min in FITC-conjugated anti-mouse secondary antibody. The cells were then mounted in paramount and viewed under a Zeiss microscope.

A: Negative Control (without anti-factor VIII); B: Cells showing Factor VIII expression.


B
A

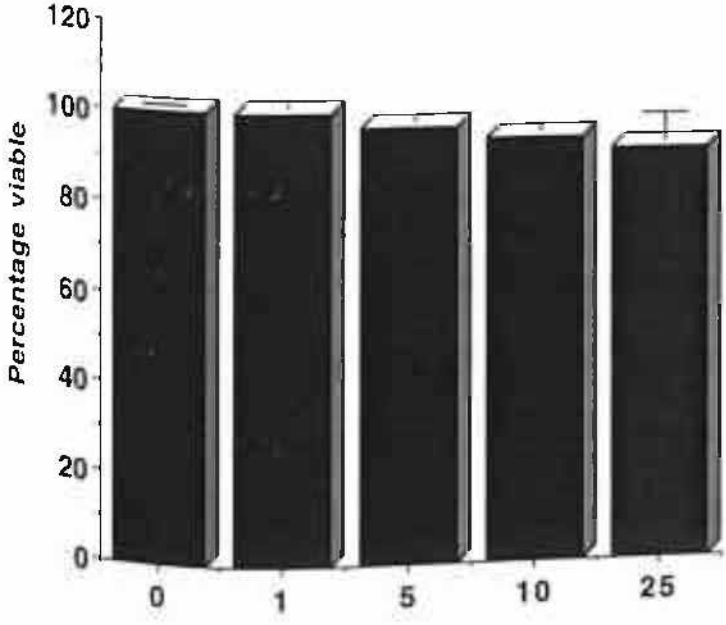
Fig.4: Effect of curcumin on proliferation: HUVEC ($2.5x10^4$) were plated in 24-well Costar plates and cultured in presence of 10% (A) or 2% (B) serum containing medium overnight. The cells were replaced with fresh medium containing 1μCi/ml ^{3}H-thymidine and various concentrations of curcumin (0-25μM) for 6h. Following treatment, the cells were washed and TCA precipitated as mentioned under Methods. The cells were lysed and the radioactivity incorporated into TCA-insoluble material was counted in a Beckamann liquid scintillation counter.


0
0
1
5
10
25
A
0
0
1
5
10
25
B

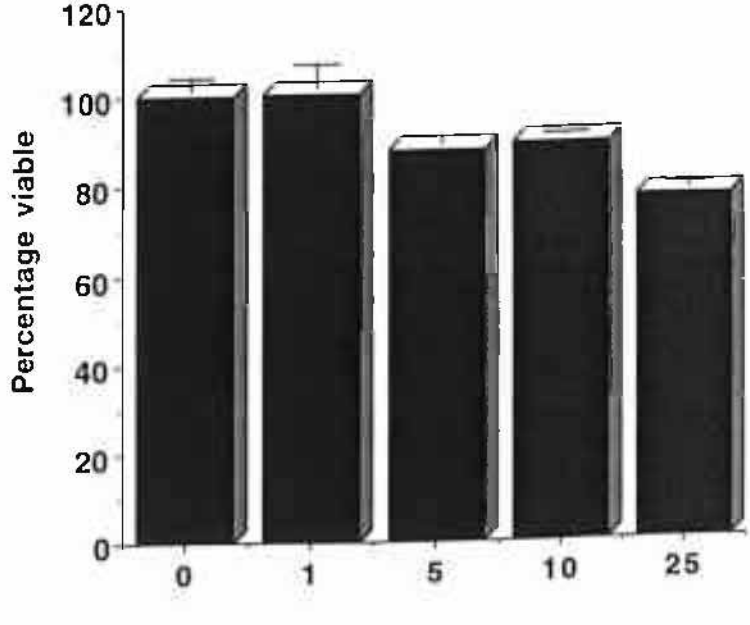
Percentage thymidine incorporation
20
40
60
80
100
120

Percentage thymidine incorporation
20
40
60
80
100
120

Fig 5: Effect of curcumin on viability: HUVEC were plated at concentrations of $3x10^4$ cells/well in 96 well plates and cultured in presence of 10% serum (A) or 2% serum (B). The cells treated with curcumin (0-25μM) for 6h were assayed for viability using MTT-kit as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN).


Percentage viable
120
100
80
60
40
20
0
0
1
5
10
25

A


Percentage viable
120
100
80
60
40
20
0
0
1
5
10
25

B

Effect of curcumin on tube formation

Treatment of HUVEC with varying doses of curcumin (1-25μM) at the time of plating on Matrigel in medium containing 10% FBS resulted in inhibition of tube formation as evidenced in Fig.6A. In presence of 10% serum, HUVEC differentiated to form an extensive network of thick tubes (Fig.6A:1). Lower doses of curcumin (1-5μM), did not markedly affect the differentiation of endothelial cells, the tubes appear to be thinner as compared to untreated control (Fig.6A:2-3) .Treatment with higher doses of curcumin (10 & 25μM) (Fig.6A:4-5), resulted in decreased tube formation and the tubes appeared to be thin and incomplete.

In order to check whether the effect of curcumin on capillary formation varies with serum concentration, we performed the tube-forming assay in presence of 2% serum. The extent of tube formation by endothelial cells in presence of 2% serum when compared to the tubes formed in presence of 10% serum is only about half-maximum (Kinsella et al., 1992). The effect of curcumin treatment on tube formation in presence of 2% serum is shown in Fig. 6B. An inhibitory effect on the network formation could be seen with as little as 1μM curcumin, although the maximal effect was observed at 25μM curcumin, where there is complete inhibition of tube formation. Treatment with a lower concentration of curcumin (5μM) resulted in incomplete and sparse tube formation (Fig.6B:3). On curcumin (10μM) treatment, the cells appear to differentiate partly, but not sufficiently to lead to tube formation (Fig.6B:4), while curcumin 25μM, lead to complete inhibition in differentiation (Fig.6B:5). These findings were quantitated as

evidenced in data Fig.7 (A&B), by assessment of the area of the culture surface covered by the tube network as mentioned under Methods. For the purpose of comparison, the tube area formed in untreated cells was assumed to be 100%, and the percentage network formation on treatment with curcumin was calculated by comparing with untreated control.

Effect of curcumin treatment at different times on tube formation

We wanted to study whether curcumin effected tube formation if the cells were treated with the compound before and after being plated on Matrigel. Since serum appears to have a protective action against the inhibitory effect on tube formation by curcumin as evidenced by our earlier experiment, we used medium containing 2% serum in all further experiments.

Pre-treatment of cells with curcumin for 3h, before plating cells on Matrigel, appeared to be the most effective as compared to all other treatment times (Fig.8A). While pre-treatment with 1μM curcumin (Fig.8A:2) demonstrated 15% inhibition in tube formation (Fig.9A), 5μM curcumin (Fig.8:A3) inhibited tubes by as much as 75%. Although curcumin at higher concentrations (10-25μM) (Fig.8:A4-5), permited cell attachment on Matrigel, it nevertheless completely inhibited their differentiation.

In order to determine the effect of curcumin or pre-formed tubes, HUVEC were plated on Matrigel and after 6h, the tubes were treated with various concentrations of curcumin. Curcumin did not affect the pre-formed tubes for almost 6h after addition, although, thereafter curcumin treatment resulted in the disintegration of the pre-formed

tubes in a dose dependent manner (Fig.8B). Prolonged incubations of HUVEC on Matrigel resulted in the thickening of the tubes which undergo further remodeling (Fig.8B:1). Treatment of tubes with 1μM curcumin did not show much change in the pre-formed tubes (Fig.8B:2), while treatment with 5 and 10μM curcumin (Fig.8B:3&4) resulted in broken tubes which appeared to lack intercellular communication. Treatment with 25μM curcumin disintegrated the tubes by almost 80% (Fig.8B:5) and the tubes appeared to shrink.

Fig.6: Inhibition of tube formation by curcumin: HUVEC ($4x10^4$cells/well) in medium containing 10% serum (A) and 2% serum (B) were plated on Matrigel pre-coated 24 well plates and treated with curcumin (0-25μM). After 6h, the cultures were fixed and stained. Curcumin treatment resulted in a dose dependent inhibition. Panel 1: Untreated control; 2:1μM Cur; 3: 5μM Cur; 4: 10μM Cur; and 5: 25μM Cur respectively.

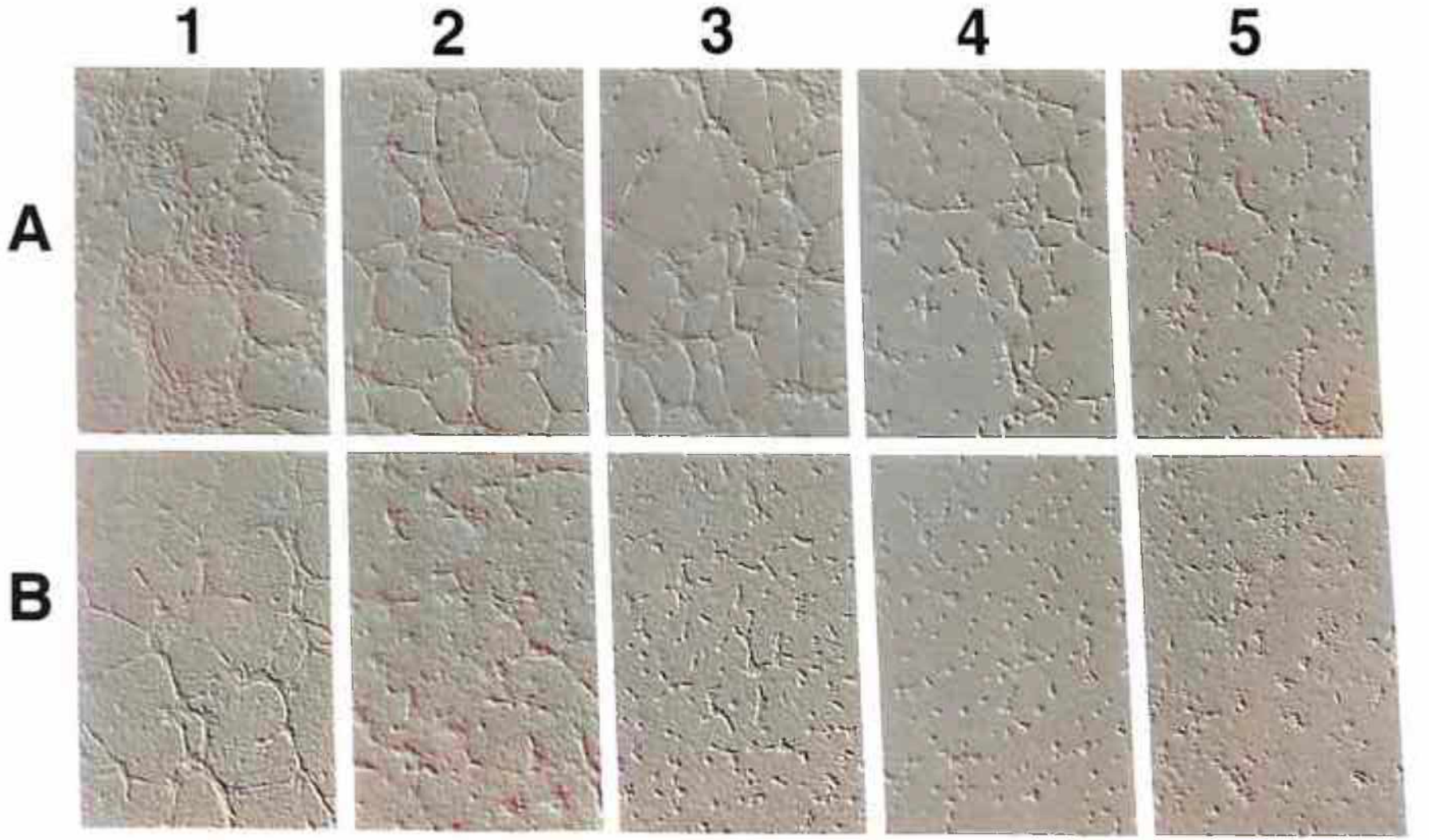
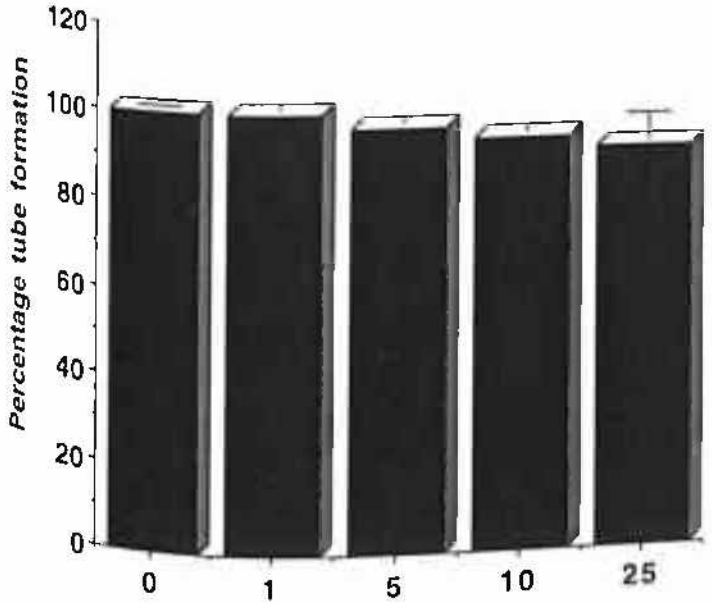
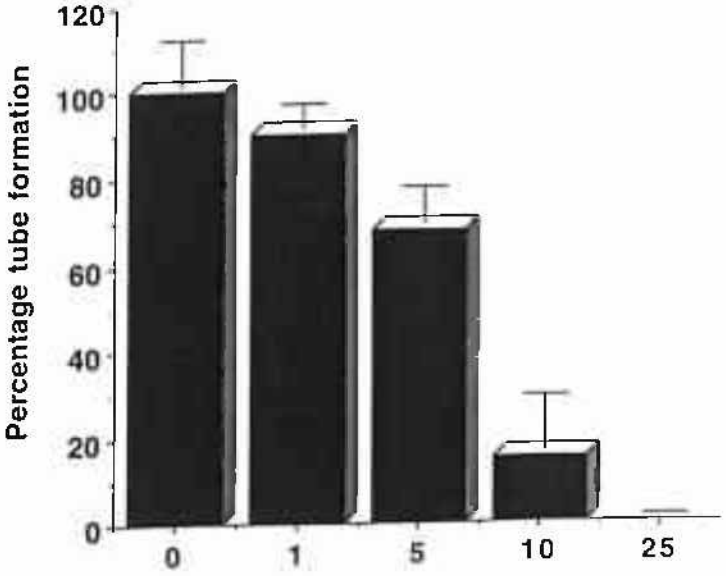

1
2
3
4
5
A
B

Fig.7: Effect of varying doses of curcumin on tube formation: HUVEC ($4x10^4$cells/well) in medium containing 10% serum (A) or 2% serum (B) were plated on Matrigel coated 24 well plates and treated with varying doses of curcumin (0-25μM) for 6h. The cultures were fixed, stained and photographed. The tubes formed were quantitated by scanning the pictures in Adobe Photoshop and NIH Image program as described under Methods.


Percentage tube formation
120
100
80
60
40
20
0
0
1
5
10
25

A

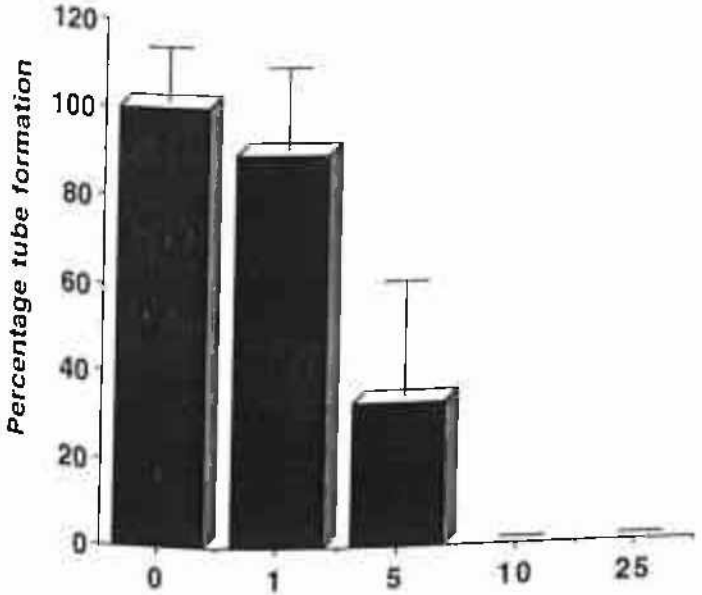

Percentage tube formation
120
100
80
60
40
20
0
0
1
5
10
25

B

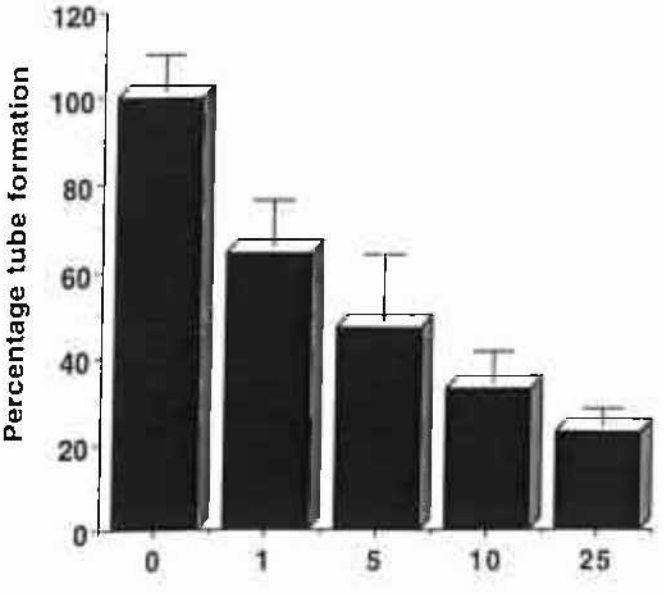
Fig.8: Effect of Pre-treatment Vs Post-tube treatment on tube formation by curcumin. (A) HUVEC were treated with varying doses of curcumin (0-25μM) for 3h. Following treatment, the cells were washed with PBS and $4x10^4$ cells/well were plated in triplicate on Matrigel- coated 24 well plates. (B) HUVEC were plated on Matrigel, and after 6h tube formation, the cultures were treated with curcumin (0-25μM), and tubes were fixed and stained after 12h. Panel 1: Untreated control; 2: 1μM Cur; 3: 5μM Cur; 4: 10μM Cur; and 5: 25μM Cur respectively.

1
2
3
4
5
A
B

Fig.9: Pre-treatment vs Post-tube treatment: Quantitation of tube formation: (A) HUVEC were pre-treated with curcumin (0-25μM) for 3h. The cells were washed, suspended in 2% serum containing medium, and plated on Matrigel. Tubes formed after 6h were fixed, stained and photographed. (B) Pre-formed tubes were treated with various concentrations of curcumin (0-25μM) and the cultures fixed after 12h treatment. The effect on tube formation was quantitated by scanning the pictures taken after fixation and staining, using NIH program.


Percentage tube formation
120
100
80
60
40
20
0
0
1
5
10
25

A


Percentage tube formation
120
100
80
60
40
20
0
0
1
5
10
25

B

Effect of curcumin on proteolytic activity

To determine the effect of curcumin on proteinases, we used a zymographic assay to visualize the proteinases expressed by HUVEC grown on plastic in the presence of 2% FBS after 6h treatemnt with different doses of curumin. HUVEC secreted three distinct types of proteinases with mol.wt. 92-kD, 72-kD and 53-kD. The gelatinolytic activity of 72-kD proteinase was much more distinct, while the activity bands produced by 53-kD and 92-kD proteinases were faint. The results of one such experiment is shown in Fig.10, which indicates a dose-dependent inhibition in the gelatinolytic activities of HUVEC by curcumin. The results also showed a greater regulation in the activities of 72-kD and 53-kD proteinases as compared to other secreted proteinases. However, when the supernatant of HUVEC grown on plastic were zymographed and incubated in curcumin containing substrate buffer, no inhibition of proteolytic activity was observed (data not shown).

Characterization of proteinases

To characterize the proteolytic activities, zymograms were further probed by incubating in the buffer containing proteinase inhibitors and the gelatinolytic activity was then determined. Results shown in Table 1 indicate that the activities of extracellular proteinases in HUVEC are specifically inhibited by certain metalloproteinase inhibitors. While incubation of gels in the presence of NEM or PMSF did not show any change in the activity of either control or curcumin-treated conditioned medium as compared to

incubation in the substrate buffer containing calcium alone, addition of EDTA or DTT completely inhibited the proteolytic activities in both the groups. Since the presence of divalent cations like Ca^{+2} are required for activating metalloproteinases, these data suggest that the proteinases regulated by curcumin belong to the class of metalloproteinases.

Effect of curcumin on the protein levels of proteinases

Conditioned media from HUVEC treated with curcumin (1-25μM) for 6h in media containing 2% serum, was estimated for protein content. Equal protein was separated on 10% SDS-PAGE. The proteins were transferred to nitrocellulose paper. Non-specific binding was blocked with 5% milk. The blot was incubated with either 72-kD, 92-kD or 53-kD monoclonal antibodies. The proteins were detected using alkaline phosphatase conjugated secondary antibody. Western analysis using 72-kD proteinase antibody showed a dose-dependent inhibition in the conditioned medium of endothelial cells treated with curcumin (Fig.11). Analysis of 92-kD and 53-kD proteinases by Western analysis did not show any signals (data not shown).

Effect of curcumin on mRNA levels of proteinases

To investigate the possible transcriptional regulation of these proteinases by curcumin, the expression of 72-kD and 92-kD genes were examined in HUVEC cultured in presence of 2% FBS containing media by assay of the corresponding mRNA steady-state levels. Northern analysis was performed after treating the cells with various

concentrations of curcumin for 3h. Curcumin decreased the expression of 72-kD and 92-kD metalloproteinase in a dose dependent manner (Fig.12). The inhibition in the mRNA transcripts of 72-kD proteinase was greater as compared to the transcripts of 92-kD proteinases.

Fig.10: Zymogram analysis of the gelatinolytic activities: Conditioned media of HUVEC treated with curcumin (0-25μM), was harvested after 6h and subjected to non-reducing SDS-PAGE through a 10% acrylamide resolving gel containing gelatin as described in the Methods. The gels were renatured and incubated overnight at 37^0C and stained with coomassie blue. Bands of gelatinolytic activityare indicated by pale bands in the gel at 92-kD, 72-kD and 53-kD. Lane on the left shows mol.wt markers.

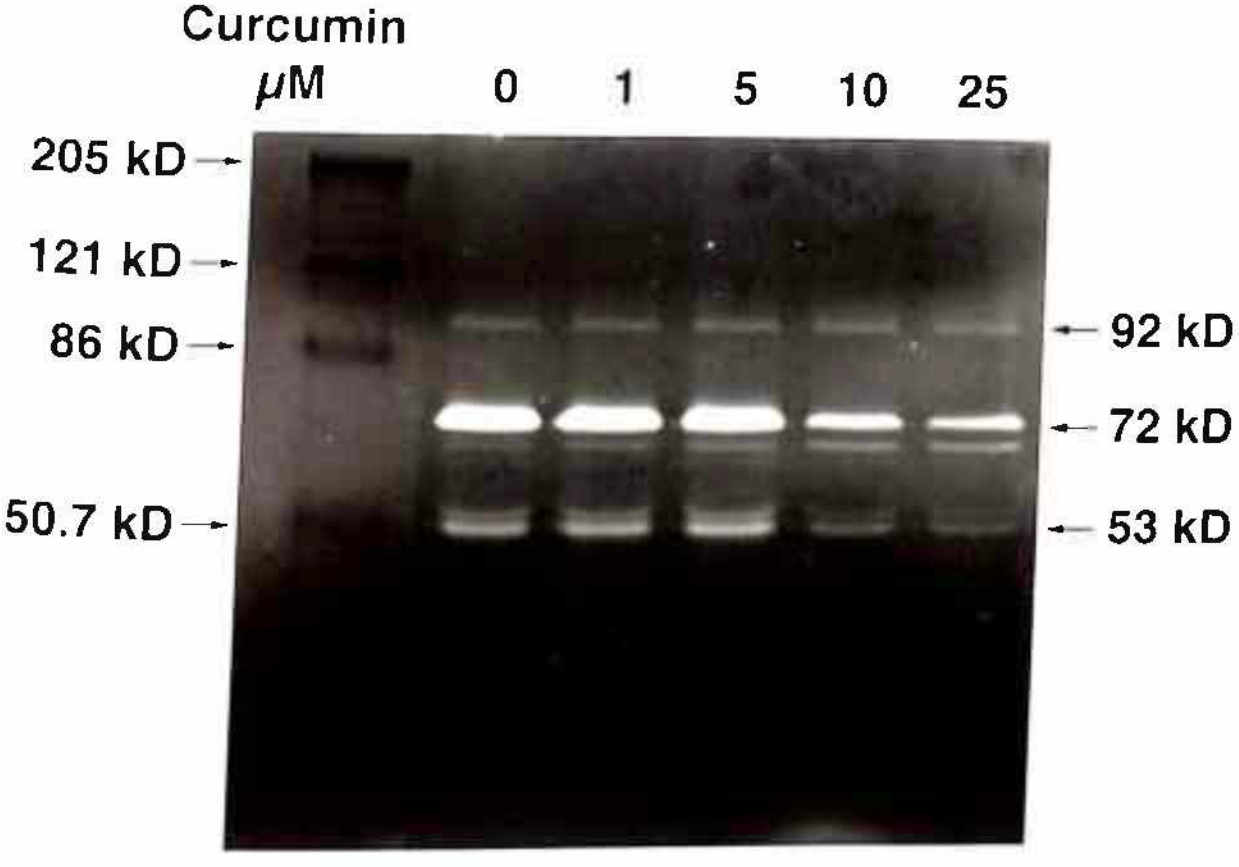

Curcumin
μM
0
1
5
10
25
205 kD
121 kD
86 kD
50.7 kD
92 kD
72 kD
53 kD

Table 1: Characterization of the proteinases

PROTE-	TREATMENT*									
	CONTROL		EDTA		DTT		PMSF		NEM	
INASES	C	Cur	C	Cur	C	Cur	C	Cur	C	Cur
~92-kD	++	+	-	-	-	-	++	+	++	+
~72-kD	++	+	-	-	-	-	++	+	++	+
~53-kD	++	+	-	-	-	-	++	+	++	+

* Conditioned medium from HUVEC treated with or without Cur 25μM was separated on SDS-PAGE as described in methods. After electrophoresis, the gels were incubated over night with either substrate buffer (control), or buffer containing EDTA (10mM), DTT (5mM), PMSF (1mM) or NEM (5mM). The gels were stained and visualized. The presence of activity is expressed as (+); absence of proteolytic activity is expressed as (-).

C-Untreated Cur- 25μM curcumin-treated

Fig.11: Northern analysis of mRNA for 72-kD and 92-kD proteinases: HUVEC grown to 75% confluency on plastic was conditioned overnight in medium containing 2% serum and treated with curcumin (0-25μM) for 3h. RNA was isolated using the RNAzol method, electrophoresed on 1% agarose gel, and probed with 72-kD c-DNA as described in the Methods. The blot was stripped and re-probed with 92-kD proteinase cDNA. Ethidium bromide stained 28S and 18S RNA show equal loading of RNA.

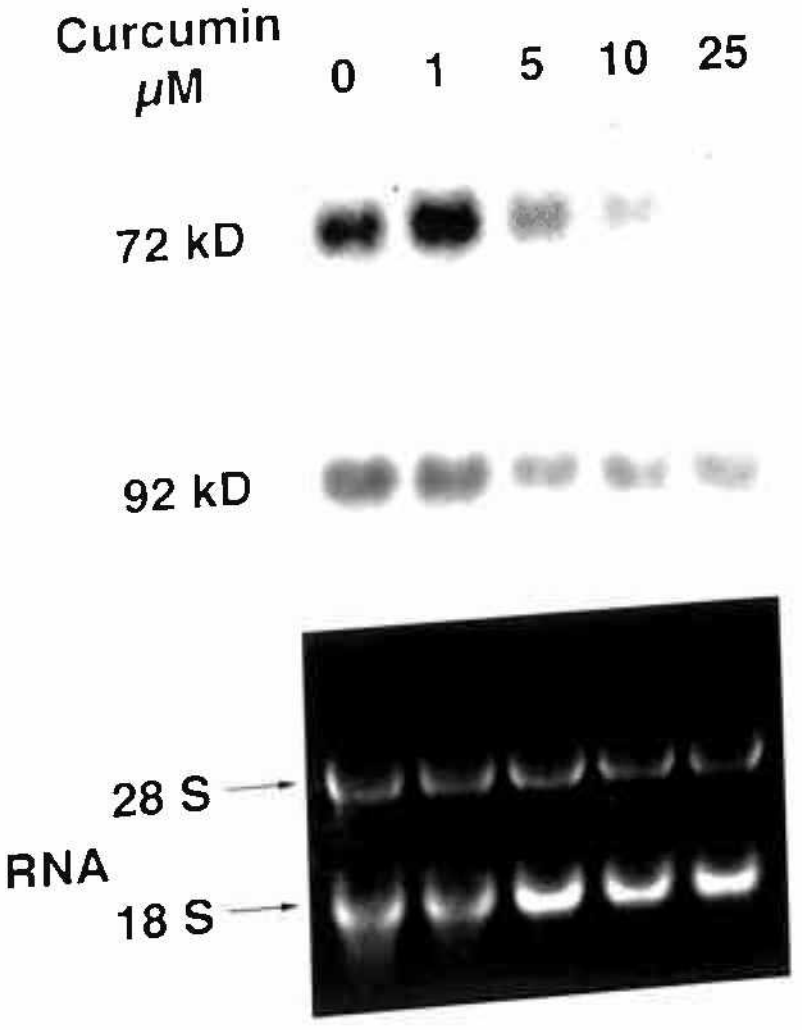
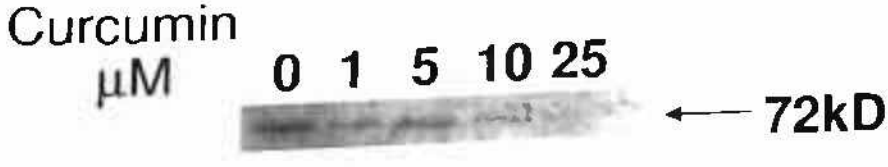

Curcumin
μM
0
1
5
10
25
72 kD
92 kD
28 S
RNA
18 S

Fig.12: Western analysis of 72-kD proteinase: Equal amounts of proteins from culture supernatants of HUVEC treated with curcumin (0-25μM) for 6h, was separated on 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal anti-72-kD antibody. The bands were developed with alkaline phosphatase-conjugated anti-mouse IgG and BCIP/NBT as described in methods.


Curcumin
μM
0 1 5 10 25
72kD

DISCUSSION

Angiogenesis is a highly regulated process that involves a complex cascade of events.Given the physiological and pathological importance of angiogenesis, extensive studies have been carried out to identify the factors that regulate this process. Growth factors such as bFGF have been shown to promote angiogenesis *in vivo* and *in vitro* by stimulating the migration, proliferation and proteolytic activity of endothelial cells (Villaschi and Nicosia,1993). The induction of tube formation in chicken chorioallantoic membrane by TGFβ is significantly different from that formed by bFGF, wherein TGF-β induced the formation of large blood vessels while FGF induced small blood vessels (Yang and Moses, 1990). In view of the therapeutic value of angiogenic inhibitors, several investigators have identified anti-angiogenic compounds. Antibiotics such as 15-deoxyspergualin *in vitro* have been shown to inhibit angiogenesis of bovine vascular endothelial cells by inhibiting cell proliferation in three-dimensional culture systems (Oikawa et al., 1992).

Since curcumin has been reported to inhibit tumor initiation (Huang et al., 1992) and tumor promotion (Huang et al., 1988), and since angiogenesis plays a major role in tumor growth and metastasis, we investigated the effects of curcumin on angiogenesis using an *in vitro* Matrigel model.

During angiogenesis, endothelial cells respond to angiogenic stimuli by migrating into the interstitium. The distal cells proliferate rapidly, thereby supplying new cells required for the developing vessels. Moses et al., (1990), have identified a collagenase inhibitor from bovine cartilage which has inhibitory action on endothelial cell migration

and cell proliferation. In general most endothelial cell proliferation inhibitors are anti-angiogenic although, their is no strict correlation between the two phenomena. For example, TGF-β has been shown to enhance the angiogenesis *in vivo*, but inhibits endothelial cell proliferation *in vitro*. (Frater-Scroder et al., 1986). TNFα and IFNγ, having tumoricidal and tumorstatic properties *in vivo* have been reported to inhibit proliferation (Friesel et al., 1987; Sato et al., 1986) and angiogenesis *in vitro* (Sato et al., 1990). Using an *in vitro* angiogenic model of Matrigel which mimics the *in vivo* angiogenic differentiation, our laboratory has shown the differential effect of interferon on tube formation (Maheshwari et al, 1991b). While interferon alpha (IFNα) enhanced tube formation of human umbilical vein endothelial cells (HUVEC), interferon-gamma (IFN-γ) has been shown to inhibit tube formation. TNF-α as well as IFN-γ inhibit angiogenesis of endothelial cells (Sato et al.,1990).

In this report, we show the inhibition of HUVEC proliferation by curcumin in a dose dependent manner. While the mechanism of this inhibition is not known, there are reports which show the inhibition of lipoxygenase and cycloxygenase by curcumin (Lysz et al., 1994). Nordihydroguaiaretic acid (NDGA), a general lipoygenase inhibitor has been reported to inhibit fetal bovine aortic endothelial cell growth (Setty et al., 1987). Recently the vital role of lipoxygenase products, in particular 12(S)-HETE (12(S)-hydroxyeicosatetraenoic acid), has been reported to control endothelial cell growth in bovine capillary endothelial cells (Dethlefsen et al., 1994). Curcumin has also been shown to be a potent and selective inhibitor of phosphorylase kinase, a key regulatory enzyme involved in glycogen metabolism (Reddy et al., 1994). It has been speculated

that this inhibition of phosphorylase kinase might block the autocrine action of growth factors needed for cellular proliferation. Kinase inhibitors have been shown to display anti-proliferative activity *in vitro* and anti-tumor activity *in vivo* (Elliot et al., 1990). Tetraphorbol acetate (TPA) has been reported to elevate the expression of oncogenes involved in cell proliferation. Recently it has been reported that curcumin inhibits TPA - induced mouse skin carcinoma in CD-1 mice by inhibiting the expression of c-fos, c-myc and c-jun proto-oncogenes (Kakkar et al., 1994). Thus, while the primary mechanism of this pleuripotent growth regulator is unknown, curcumin has pleiotropic effect in common with other growth regulating compounds.

Curcumin does not affect the viability of endothelial cells at low doses inspite of proliferation being greatly inhibited. The proliferation of HUVEC is completely inhibited by curcumin (>5μM), even on 6h treatment. The cells resume growth slowly after the removal of curcumin. This correlates well with nutritional reports that indicate that despite being eaten in moderate doses for many centuries, curcumin has not been reported to be toxic (Ammon & Whal,1991). It has been shown that the effective concentration of Suramin, an anti-neoplastic agent, required to inhibit cell proliferation in presence of 10% serum was much higher than in presence of 2% serum (Ewing et al.,1993). Our observation of complete cell viability in 10% serum and partial viability in 2% serum indicated that the effect of curcumin on cell growth was similar to Suramin. Since serum is a source of several growth factors, it may be counteracting the effects of curcumin by stimulating the expression of growth promoting genes. Thus, while the inhibition of proliferation is more effective at 2% serum concentrations, the concentration

that this inhibition of phosphorylase kinase might block the autocrine action of growth factors needed for cellular proliferation. Kinase inhibitors have been shown to display anti-proliferative activity *in vitro* and anti-tumor activity *in vivo* (Elliot et al., 1990). Tetraphorbol acetate (TPA) has been reported to elevate the expression of oncogenes involved in cell proliferation. Recently it has been reported that curcumin inhibits TPA - induced mouse skin carcinoma in CD-1 mice by inhibiting the expression of c-fos, c-myc and c-jun proto-oncogenes (Kakkar et al., 1994). Thus, while the primary mechanism of this pleuripotent growth regulator is unknown, curcumin has pleiotropic effect in common with other growth regulating compounds.

Curcumin does not affect the viability of endothelial cells at low doses inspite of proliferation being greatly inhibited. The proliferation of HUVEC is completely inhibited by curcumin (>5μM), even on 6h treatment. The cells resume growth slowly after the removal of curcumin. This correlates well with nutritional reports that indicate that despite being eaten in moderate doses for many centuries, curcumin has not been reported to be toxic (Ammon & Whal,1991). It has been shown that the effective concentration of Suramin, an anti-neoplastic agent, required to inhibit cell proliferation in presence of 10% serum was much higher than in presence of 2% serum (Ewing et al.,1993). Our observation of complete cell viability in 10% serum and partial viability in 2% serum indicated that the effect of curcumin on cell growth was similar to Suramin. Since serum is a source of several growth factors, it may be counteracting the effects of curcumin by stimulating the expression of growth promoting genes. Thus, while the inhibition of proliferation is more effective at 2% serum concentrations, the concentration

of curcumin required for the same degree of inhibition in presence of the 10% serum is higher. Growth factors are known to induce proliferative genes such as c-fos and c-myc which are inhibited by curcumin. Higher doses of curcumin overcome the effects of serum which is consistent with this explanation. Growth factors are known to induce proliferative genes such as c-fos, c-myc and c-jun which are inhibited by curcumin (Kakkar and Roy, 1994). Higher doses of curcumin however, overcome the effects of serum which is consistent with this explanation.

Treatment of HUVEC with non-toxic doses of curcumin resulted in the inhibition of the differentiation of HUVEC on Matrigel. The degree of inhibition differs with serum concentration, which is similar to its effect on cell viability and proliferation. However, in contrast to its effect on cell viability, where its effect is only partial curcumin inhibits angiogenic differentiation completely. The effective concentration required to inhibit angiogenesis in presence of 10% serum is much higher as compared to 2% serum. Kinsella et al., (1992), have shown a serum-dependent difference in tube formation on Matrigel. As 2% serum containing media is sufficient for endothelial cells to form tubes on Matrigel, we performed angiogenic experiments in 2% serum containing media. The inhibition was seen at all doses irrespective of the time it was added to the cells. The effects of curcumin may be irreversible or slowly reversible since the differentiation of HUVEC is still inhibited when the cells are pre-treated with the drug for 3h and then extensively washed before plating on Matrigel. In fact, pre-treatment is more effective in preventing tube formation since complete inhibition was observed at a concentration of 10μM, whereas a higher concentration (25μM) was required for complete inhibition

when cells are treated at the time of plating on Matrigel. This effect of curcumin may be due to its regulation of early genes involved in differentiation, since it has been reported to regulate the expression of many growth regulating genes. Endothelial cells plated on Matrigel undergo continuous remodeling, which ultimately leads to the shrinkage of tubes. This was evidenced by incubation of the cells on Matrigel for 16-18h which lead to changes in the tube formation as compared to early time points. Curcumin treatment of pre-formed tubes resulted in the disintegration of tubes. This may be due to the inhibition of further differentiation and remodeling, which ultimately leads to the shrinkage of tubes. The inhibition of differentiation in HUVEC by curcumin may not be related to cell cycle events since even fully differentiated tubes are disrupted by low doses of curcumin at concentrations at which the viability of cells grown on plastic is not at all affected.

Kinsella et al., (1992), have shown that the tube formation induced by PMA can be blocked by protein kinase C (PKC) inhibitors, thus, implicating the possible role of PKC activation by PMA in regulating the formation of tube structures. Curcumin has been reported to partially inhibit PKC (Reddy et al., 1994) and treatment of TPA stimulated NIH3T3 cells with curcumin resulted in inhibition of TPA-induced PKC activity in both cytosolic and particulate fractions, but did not affect the level of PKC protein (Liu et al,1993). Quercetin, an anti-oxidant and lipoxygenase and cyclooxygenase inhibitor like curcumin is a potent tumor suppressor and shows inhibitory effects on PKC activity (Gschwendt et al.,1984). The suppression of PKC activity by curcumin could also be involved in inhibiting angiogenesis. In contrast to HUVEC plated on plastic wherein, the cells have a cobblestone morphology and are highly proliferative, HUVEC plated on

Matrigel exhibit inhibition of proliferation and promotion of differentiation (Kuzuya and Kinsella,1994). Therefore, the effect of curcumin on proliferation observed on plastic cannot be correlated to its inhibitory effect on Matrigel as no proliferation occurs on Matrigel.

One event that plays a key role in angiogenic process is the re-organization of extracellular matrix which is a complex and dynamic network assembled outside of the cell using specific secreted glycoproteins and proteoglycans. Matrix proteolysis plays a critical role in several stages of tumor development, including the process of angiogenesis, which is critical for tumor development. Tumor-promoting phorbol esters have been reported to induce the infiltration of bovine microvascular endothelial cells into collagen matrix by increasing the secretion of proteinases like collagenase and plasminogen activator (Montesano and Orci,1985). This effect of PMA on endothelial infiltration could not be blocked by a spectrum of inhibitors active against serine, thiol, and carboxyproteinases but could be prevented by the metalloproteinase inhibitor 1,10-phenanthroline, thereby implicating a possible role of metalloproteinases (eg. collagenase) in the invasion process. Although phorbol esters are not physiologically occuring substances, they cause a variety of effects in cultured cells, many of which are also elicited by hormones or growth factors. Angel et al., (1987), have examined the 5'upstream sequences of human collagenase genomic c-DNA and identified the phorbol responsive element (TRE) as the AP-1 binding site. As c-fos and c-jun are among the protein components of AP-1, induction of c-fos is essential for phorbol induction of collagenase. The requirement for c-fos in collagenase induction by oncogenes has been demonstrated by Schonthal et al., (1988), by the use of a plasmid to transfect cells with

anti-sense fos sequences, which completely abolished the collagenase response. Thus curcumin, a potent inhibitior of the transcriptional factor c-jun/AP-1 (Huang and Lee et al., 1991) may be inhibiting the transcription of collagenases.

Recent reports (Schnaper et al.,1993) indicate that metalloproteinases and their inhibitors, TIMPs play a major regulatory role in matrix re-organization and the initiation of angiogenesis. We examined the effect of curcumin on the expression of proteinases to see whether curcumin may inhibit angiogenesis by regulating the expression of these key molecules. The conditioned media from HUVEC plated on Matrigel and treated with curcumin did not show significant changes in proteolytic activity. This could be explained on the basis of the interference caused by the presence of endogenous metalloproteinases in Matrigel (Mackay et al., 1993). We therefore, decided to study the regulation of metalloproteinases by curcumin in cells grown on plastic in order to avoid the complications arising from the presence of the proteolytic enzymes in Matrigel. Further, we have seen that curcumin did not inhibit the proteolytic activity when the conditioned medium from untreated cells were incubated in substrate buffers containing curcumin. In our experiments we have observed a dose-dependent inhibition of proteinases on treatment with curcumin. The properties of the proteinases are consistant with them being neutral proteinases. The enzyme activities were not inhibited by serine nor cystine proteinase inhibitors, but were inhibited by DTT; suggesting a divalent cation requirement for enzymatic activity as evidenced by inhibition of proteolytic activity when treated with EDTA. Thus, the proteinases activities regulated by curcumin appear to belong to the metalloproteinases group. The preference of the enzymes for gelatinase substrate has been evidenced by its inability to degrade caesin substrates (data not

shown).

The biochemistry of metalloproteinase activation has been well characterized through many *in vitro* studies. The mechanism for mammalian metalloproteinase activation has been referred to as the "cystein switch", whereby an unpaired cystein residue in the profragment co-ordinates with the active site zinc atom and maintains the latency of the enzyme. When this cystein-zinc atom interaction is interrupted by chemical or physical means, a conformational change occurs and subsequent proteolytic cleavage of the amino-terminal profragment ensues (Birkedal-Hansen et al.,1993). SDS-substrate gel analysis proved to be a powerful tool for studying metalloproteinases activity. The proenzymes secreted by HUVEC exhibited proteolytic activity in the gels, because as reported by Chin et al.,(1985), SDS interrupts the cystein-zinc bond thereby activating proenzymes, collagenase and stromelysin. HUVEC synthesized at least three different proenzyme species, all of which were regulated by curcumin in a dose dependent manner. The level of inhibition of 72-kD and 53-kD proteinases was more pronounced with 10 and 25μM treatment showing maximum inhibition as compared to lower concentrations. By far, the most extensive degradation by substrate gel analysis was observed for the lysis zone centered at 72-kD and 53-kD. Similar metalloproteinases have been detected from rabbit brain capillary endothelial cells by Herron et al., (1986). The authors have demonstrated the presence of proteolytic activity in rabbit capillary endothelial cells only when stimulated with TPA. However, we show HUVEC to secrete metalloproteinses without induction by phorbol esters.Our experiments to check the inhibition of activity of proteinases by curcumin as well as lack

of regulation of proteolytic activity by curcumin from conditioned media of HUVEC on Matrigel indicated that curcumin does not inhibit the activity but could be regulating the expression of the metalloproteinases at the transcriptional or translational level. However, Schnaper et al., (1993) have shown that the early expression of metalloproteinases are involved in tube formation. Therefore, it appears that curcumin could be regulating the expression of metalloproteinases. On plastic, curcumin differentially regulated the expression of 72-kD and 53-kD proteinases. While the inhibition of 72-kD proteinase at the protein level is clearly observed by Western blotting, regulation of 53-kD and 92-kD proteinase levels could not be detected. This could be due to the lack of sensitivity in the protocol. While Northern analysis demonstrated regulation of 72-kD and 92-kD proteinases, the extent of inhibition at the mRNA levels was higher in 72-kD metalloproteinase as compared to 92-kD metalloproteinase. From our observation we report that curcumin inhibits angiogenesis as well as regulates the RNA transcipts of 72-kD and 92-kD proteinases. However, the extent of inhibition of metalloproteinase expression does not correlate with the extent of inhibition of angiogenesis by different doses of curcumin. Thus, the effect of curcumin on angiogenesis may be partly due to its regulation of proteinase expression. Inhibitors of cellular protein kinases have been shown to be involved in the regulation of angiogenesis (Kinsella et al.,1992; Wright et al., 1992; Tsopanoglou et al., 1994). Curcumin, being a partial inhibitor of protein kinase activity (Liu et al., 1993; Reddy and Aggarwal, 1994), may also regulate angiogenesis by regulating protein kinase activities. Since curcumin exhibits pleiotropic effects, changes in the expression of metalloproteinases may be one of the mechanisms by which it could be inhibiting

angiogenesis. While the mode of action of curcumin is not completely understood at present, we are pursuing further studies in our laboratory to probe this mechanism.

EFFECT OF CURCUMIN ON WOUND HEALING

INTRODUCTION

Wound healing is a complex series of biological events that in many ways resembles the process of development. In simple organisms repair is essentially the regeneration of portions of cellular structure, but in more complex organisms, the regeneration of connective tissue, vasculature, and covering epithelium becomes the predominant reaction to injury. Cellular proliferation, migration, differentiation, and tissue remodeling occur not only during embryonic and postnatal development but also recur during wound healing. Injury to skin initiates a cascade of events that follow a biological time-table. The basic responses and their regulatory mechanisms are localized to the immediate vicinity of injury. The tissue response to injury has been divided into three overlapping phases namely, inflammatory phase, proliferative phase, and remodeling phase. The different pathways involved in wound healing is shown in Fig.13

Inflammation

During the repair process after wounding, blood vessel disruption results in extravasation of blood constituents, platelet aggregation, blood coagulation and bradykinin production. In response to this, inflammatory connective tissue and vascular elements appear in the damaged area in a specific sequence. Their concerted function eventually leads to the creation of a healed wound. The leakage of plasma and other blood elements from damaged vessels results in clotting.

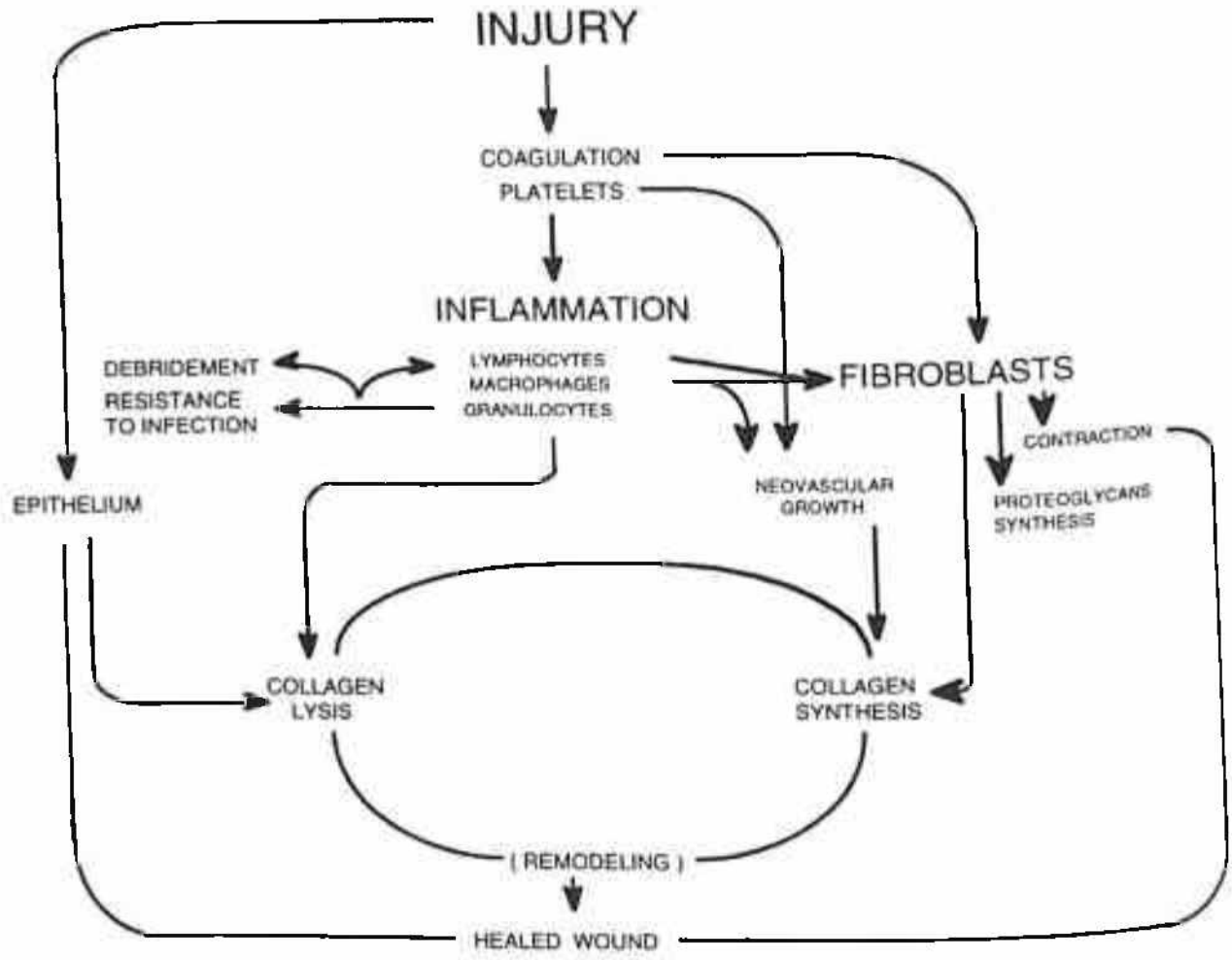


Fig.13: Schematic representation of the pathways of wound repair

Several intrinsic activities of intact blood vessel endothelium limit the clot to the damaged areas of vasculature. While the blood clot within the vessel lumen maintains hemostasis, the coagulum within the wound space provides the matrix for cell migration. For example, fibrin along with fibronectin serves as a matrix for the influx of monocytes and fibroblasts (Clark, 1993).

Neutrophils followed by monocytes infiltrate into the wound area. Neutrophils in response to chemotactic factors produced by products of the coagulation cascade, prevent infection by phagocytizing and killing microorganisms and lysing devitalized tissue by the release of proteases (Sato et al., 1994). These neutrophils are short lived lasting only several hours after digesting bacteria and necrotic tisssue. However, the presence of neutrophils in the wound environment is not critical, as neutropenia does not interfere with wound healing (Clark, 1988).

Monocytes are initially attracted to the wound site by the same stimulus as for the neutrophils, and their recruitment continues through signals released by monocyte specific chemoattractants like the extracellular degradation products, thrombin and transforming growth factor-beta (TGF-β) (Norris et al., 1982; Kingsnorth & Slavin, 1991). The migrated monocytes differentiate into macrophages (Diegelman et al., 1981), kill bacteria, produce vasoactive mediators and direct subsequent wound healing by releasing a series of growth factors like (TGF-β), transforming growth factor-alpha ($TGF\alpha$), platelet derived growth factor (PDGF) and insulin like growth factor-1 (IGF-1) (Hosgood, 1993). These cytokines are important in inducing cell migration and proliferation as well as matrix production.

The most important cell immediately after injury is the platelet. Platelet is the first cell at the site of injury, appearing almost immediately, coinciding with hemorrhage (Ford et al., 1989). The loss of the endothelial integrity of the vessel during tissue damage results in the exposure of type IV and V collagen in the sub-endothelium. This promotes aggregation and binding of platelets to these structural proteins and results in platelet activation. The activated platelets exert their inflammatory effects through the release of a number of growth factors, including PDGF, TGF-β and epidermal growth factor (EGF) (Clark, 1993). These cytokines are thought to play an important role in the wound healing process, since they function as growth factors that stimulate the proliferation of non-inflammatory cells after their migration into the wounded area (Clark, 1993). For the most part, secretion of growth factors by inflammatory cells is dependent upon activation. Once activated by infection agents, other antigens, or upon tissue injury, the cells generate numerous factors that promote local inflammation as well as recruitment, proliferation and function of fibroblasts and endothelial cells (Hunt, 1991). As both T-lymphocytes and macrophages produce cytokines capable of regulating endothelial cell and fibroblast growth and function, these cytokines contribute to the link between inflammation and repair. Anti-inflammatory agents, in particular, corticosteroids, given before wounding or during the crucial 1 to 3- day inflammatory phase, will markedly attenuate this response, and in turn, the subsequent cytokine directed response of mediators and the inflammatory cells. Vitamin A has been found to counter the anti-inflammatory effect of corticosteroids on the wound (Stephen et al., 1971). Adriamycin (doxorubicin), an effective anti-neoplastic agent administered in rats has been found to

impair wound healing (Devereaux et al., 1979). This inhibition has been demonstrated to be reversed on treatment with a combination of growth factors like PDGF, TGF-β and EGF in a wound chamber model (Lawrence et al., 1986).

Cellular proliferation

One of the major goals of the body during cutaneous wound repair is the reconstitution of the skin as a functional barrier. The epidermis reacts to the defect caused by injury within 24h. The keratinocytes initially respond to the presence of an epidermal defect by migrating from the free edge of the wound. An early provisional matrix formed by fibrin, fibronectin and type V collagen enables keratinocytes to migrate into the wound (Hammar, 1993). Growth factors like TGF-β stimulates keratinocyte production of fibronectin and its deposition in the extracellular matrix (McKenzie et al., 1990). TGF-β also stimulates epidermal migration. Induction of extracellular matrix (ECM) gene expression in keratinocytes by TGF-β might be responsible for epithelial regeneration observed during wound healing process (Vollberg, 1991)

Angiogenesis

For the wound to heal sufficient blood flow is required to allow delivery of nutrients. Endothelial cells are the most important cells involved in angiogenesis. Macrophages exposed to decrease in the surface tissue oxygen tension secrete a substance of 2000 to 20,000 MW, known as angiogenesis factor, which is considered

to be a chemoattractant for mesothelial and vascular endothelial cells (Banda et al., 1982). Endothelial cells proliferate and form capillaries at the wound surface in response to growth factors like FGF released by macrophages and platelet signals, or due to low oxygen tension, biogenic amines and lactic acid occuring in wounds which lead to the injury of the parenchymal cells, thereby causing the release of FGF (Clark, 1993). The family of FGF interact with heparin and enhance new vessel growth (McGrath, 1990). These capillaries form a network of loops which fuse with each other to form a new capillary bed. Villaschi and Nicosia (1993), have shown that injury to rat aorta leads to the release of FGF, thus providing the chemical signals required for the formation of microvessels.

Fibroblast proliferation

Fibroblast begins to appear in the wound about two days after injury. The initial cells migrate from nearby connective tissue (Carrico et al., 1984). Expasion of the fibroblast population is not only as a result of recruitment of fibroblasts at the lesion, but also the consequence of proliferation owing to the locally generated fibroblast growth factors. Macrophages, fibroblasts and blood vessels move into the wound space as a unit, which correlates well with the proposed biologic dependence of the cells during tissue repair. While macrophages provide a continuous source of cytokines necessary to stimulate fibroplasia and angiogenesis, fibroblasts construct new ECM necessary to support cell in-growth, and blood vessels carry oxygen and nutrients necessary to sustain cells and metabolism. Multiple complex interactive biologic phenomena occur

within fibroblasts as they respond to wound cytokines, including the induction of additional cytokines. Regardless of their exact origin, cytokines generated at a wound site act in concert to induce fibroblast proliferation and migration into the wound space. Pierce et al., (1989) have shown TGF-β and PDGF to be the most important stimulants for tissue repair. The fibroblasts are activated by direct stimulation through TGF-β released from the macrophages and also secondary to endothelial stimulation by TGF-β and bFGF. Macrophages secrete tumor necrosis factor-alpha (TNF-α), besides other growth factors mentioned earlier. TNF-α promotes fibroblast proliferation (Sugarman et al., 1985), collagen and collagenase biosynthesis and prostaglandin release (Beutler et al., 1986). Although TNF-α is an inhibitor of vascular endothelial cell growth *in vitro*, it promotes angiogenesis and new blood vessel formation *in vivo* (Frater-Schroder et al., 1987), contributing to the reparative phase of inflammation.

Connective tissue formation

As fibroblasts migrate into the wound space, their endoplasmic reticula and golgi apparatus begin to disperse throughout the cytoplasm and begin to deposit loose ECM composed of large quantities of fibronectin (Pierce et al., 1989). The fibronectin matrix not only enhances fibroblast migration but also provides a scaffold for collagen fibrils and mediates wound contraction. As angiogenesis proceeds with the formation of new vessels and increased oxygen capacity, this stimulus for fibroblast proliferation diminishes and undergoes a series of phenotype switch to a profibrotic phenotype, during which they produce abundant type I and III collagen (Welch et al., 1990).

Subsequently during wound contraction, the fibroblasts begin to assume the phenotype of myofibroblasts, which are characterized by large bundles of actin-containing microfilaments. Fibroblasts bind to fibronectin, vitronectin and fibrin through specialised cell membrane receptors or integrins (Ruoslahti, 1991). Fibroblasts effect the extacellular matrix through new synthesis, deposition and remodeling of the ECM (Kurkinen et al., 1980), whereas the ECM affects fibroblasts by regulating their function, including their ability to synthesize, deposit and remodel ECM (Mauch et al., 1988). The new connective tissue also contains glycosaminoglycans and proteoglycans. The synthesis of these matrix proteins occurs concomitantly with the production of new collagen.

Wound contraction

The contractile forces produced by granulation tissue in wounds are derived from myofibroblasts that contain contractile proteins. Myofibroblasts are the most numerous cells in mature granulation tissue and are aligned within the wound along the lines of contraction (Skalli and Gabbiani, 1988). F-actin bundle arrays, cell-cell and cell-matrix linkages, and collagen cross-links are involved in wound contraction. Addition of PDGF to cultured fibroblasts lead to the contraction of collagen matrix (Clark et al., 1989). The PDGF present in the wounds could be involved in providing the signal for wound contraction.

Remodeling phase

This phase involves the deposition of matrix materials and their subsequent change over time. Dermal macromolecules such as fibronectin, hyaluronic acid, proteoglycans and collagen are deposited during repair and serve as a scaffold for cellular migration and tissue support (Kirsner et al.,1993). Even after the skin is restored, events continue to occur that are related to wound injury and repair. The total amount of collagen increases early in the repair process, reaching a maximum between two and three weeks after injury. During the remodeling phase, collagen III which was deposited in the dermis in the early stages of wound healing is converted to type I collagen. This conversion is accomplished through tightly regulated interactions between synthesis of new collagen and lysis of old collagen brought about by collagenases (Kanzler et al., 1986).

Role of growth factors in wound repair

Growth factors are produced by different cells aiding in wound healing, and are affective during replacement and reconstitution. These are termed as competance or progression factors, depending upon the ability to initiate the cell into mitosis or initiate the machinery of mitosis respectively. The accumulation of platelets at the wound site in response to injury initiates the cascade of growth factors present in the wound via the release of PDGF, TGF-β and EGF (Kudlow et al., 1987). PDGF is believed to direct the sequential migration of monocytes, neutrophils and fibroblasts to the site of wound

repair. Activation and proliferation of early wound cells results in endogenous production of growth factors which in turn act as chemoattractants for other inflammatory cells to secrete regulatory growth factors or to migrate to the site of tissue repair to modulate the growth environment. Fibroblasts, keratinocytes, endothelial cells and lymphocytes migrate into the site after the initial inflammatory reaction, providing a further cascade of growth factors, cell proliferation and extracellular matrix deposition. Wound healing therefore requires a complex orchestration of different cell types and regulation by growth factors and other cytokines.

Transforming growth factor- beta (TGF-β)

This is a low-molecular weight protein with a broad array of biological effects. Sporn et al., (1983) have shown the effect of TGF-β *in vivo*, by the addition of partially purified TGF-β into the Schilling-Hunt wound chamber implanted into the back of rats. The analysis of the contents of the wound chamber two weeks after insertion, showed increased collagen deposition and fibroblasts infiltration thereby implicating the role of TGF-β in wound healing. This effect has been further proved by Lawrence et al., (1986) who have demonstrated the ability of TGF-β to reverse the inhibition of wound healing by Adriamycin in rats. They have shown by collagen content, protein content, cellular proliferation rate and histology that TGF-β reversed most of the Adriamycin-induced wound-healing impairment. Also, of all the growth factors like PDGF, EGF, and TGF-β tested, TGF-β most effectively reversed the Adriamycin-induced impairement. The effect of TGF-β has been reported to be potentiated by the presence of PDGF and EGF;

together, these three peptides reversed almost 90% of Adriamycin-induced impairement. Injection of purified TGF-β into new born mice by Roberts et al., (1986) led to the formation of granulation tissue at the site of injection. Morphological studies of the tissue showed that angiogenesis and collagen deposition were induced by TGF-β. Fibroblasts, macrophages and granulocytes were observed at the injection site. Similar studies with saline or EGF however, did not induce the same response, thereby showing that the *in vivo* presence of TGF-β is closely associated with wound-healing response.

The induction of integrins like $\alpha_5\beta_1$ and $\alpha_v\beta_3$ by TGF-β on cultured fibroblasts as well as keratinocytes have been reported to facilitate their migration into the wound area during re-epithelialization (Gailit et al., 1994). Garlick et al., (1994), using an organotypic co-culture have demonstrated a dose dependent inhibition of proliferation of keratinocytes and delay in re-epithelialization by TGF-β. This delay in re-epithelialization has been attributed to the modification of proliferation and migration by TGF-β.

Epidermal growth factor (EGF)and Transforming growth factor-alpha (TGF-α)

EGF is a polypeptide composed of 53 amino acids. TGF-α shares a 30% amino acid similarity with EGF and binds to EGF receptor. Martin et al., (1992) have shown the presence of TGF-α in skin keratinocytes and activated macrophages, and binds to EGF receptors. Both EGF and TGF-α have been identified at the wound site after injury. The requirement of EGF in collagen formation, granulation tissue development, and

enhancing epithelialization as demonstrated in animal models suggests that EGF influences both fibroblasts and epithelial cells which are known to play an important role in wound healing. Topical application of EGF and TGF-α separately on burn wounds showed increased epithelialization by both growth factors (Brown et al., 1989). TGF-α, however, was seen to be more potent than EGF.

Platelet derived growth factor (PDGF)

PDGF is a glycoprotein consisting of two chains, α and β, and are 60% homologous with one another. This growth factor arrives at the wound site from several sources, including degranulated platelets, activated macrophages, and smooth muscle cells of vascular endothelium. The presence of PDGF is known to increase fibroblasts and inflammatory cell infiltration, as well as collagen and granulation tissue production (Herndon et al.,1993). The stimulation of fibroblasts by PDGF leads to the secretion of collagenase which may be involved in the removal of damaged collagen tissue or in the remodeling of tissues.

The role of ECM in wound repair

The importance of ECM in wound repair is shown in *Fig.14*. The diverse extracellular matrices present in ECM are uniquely capable of modulating cellular behaviour and phenotype. By binding specific cytokines and growth factors, ECM sequesters potent biological response modifiers in the environment of the healing wound and aids in organizing the geometric framework that facilitates cell migration and

modulate cell-cell interactions (Raghow, 1994). The dynamic cell-ECM interactions are continuously modified by a host of potent cytokines, and are subject to feedback regulation mediated by transcription, post-transcriptional, translational, and post-translational mechanisms during wound repair.

Fibronectin

Fibronectins are modular cell-adhesive and matrix-organizing glycoproteins that aid in healing by stimulating cell attachment and migration by forming part of the initial connective tissue matrix at wound sites (McDonald, 1988). TGF-β and bFGF activate keratinocytes, and TGF-β stimulates the keratinocytes to produce fibronectin, an attachment filament promoting spreading. Fibronectin induces proliferation indirectly by the release of TGF-α/EGF from keratinocytes in an autocrine fashion. During migration, keratinocytes degrade fibronectin, reconstitute their fibronectin receptors, and produce new material for further attachmant (Clark, 1990). In the skin normally, fibronectin is not expressed in the ECM produced by the fibroblasts. However, once a wound has occured, the transient appearance of fibronectin is detectable in the ECM first. The keratinocytes then develop receptors for this fibronectin and respond to it with increased motility. The keratinocytes move along with this fibronectin trail to help close the wound and, with the closure of the wound, the production of fibronectin by the fibroblasts ceases, the keratinocytes looses their receptors for fibronectin (Mac Neil, 1994).

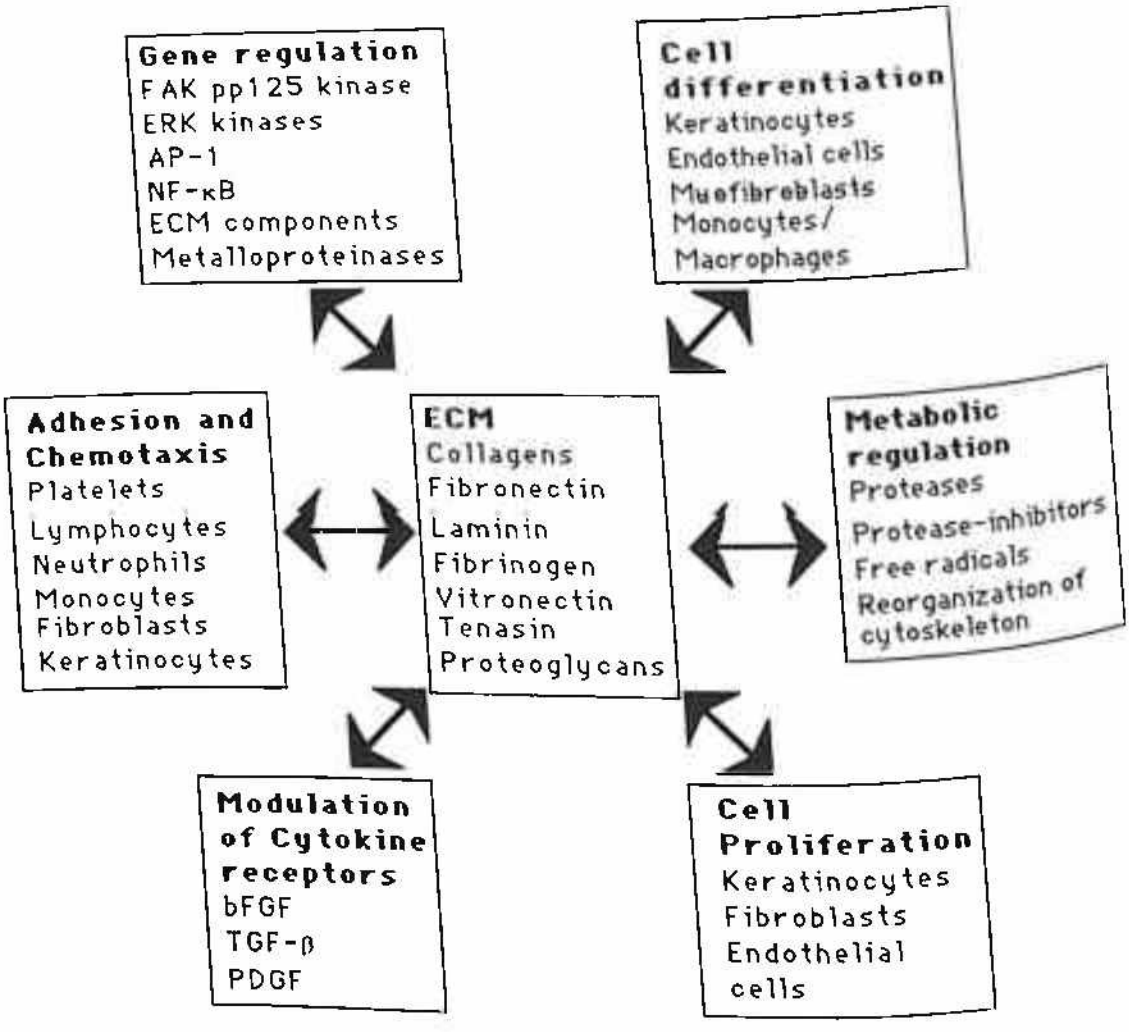


Fig.14: Schematic representation of the role of ECM in wound healing

Collagens

Collagens form the backbone of ECM, and based on their source, these are classified as fibrillar (type I, II, III), non-fibrillar (type IV) and FACIT collagens (fibril associated collagens with interrupted triple helices). Collagen I, III and IV have been implicated in wound healing and tissue regeneration. The involvement of fibrilar collagen in platelet aggregation during the early stages of wound healing has been reported by Shoshan et al., (1981). The subsequent binding of platelets to the fibrillar collagen, leads to the release of several large glycoproteins such as fibronectin and thrombospondin. During granulation, tissue formation which begins three to five days after wounding and continues for ten to twelve days shows rapid synthesis of type I and III collagen and associated increase in tensile strength in cutaneous wounds of the rat (Madden and Peacock, 1968). The type I and III collagen in conjuction with non collagenous proteins such as fibronectin provides support for epidermal cell migration and proliferation. The factors regulating collagen production can be divided into those that participate in the early, intermediate, and later stages of the process. The tensile strength is provided to the wound by the aggregation of collagen macromolecules into fibrillar bundles (Clark, 1993). The transition of granulation to mature scar depends on collagen synthesis as well as collagen catabolism. The degradation of the wound collagen is controlled by a variety of collagenase enzymes secreted by fibroblasts, macrophages and granulocytes (Hasty et al., 1986). Besides providing structural support and strength to the new tissue, collagen can have a profound effect on the cells within and on its matrix. Collagen and collagen derived peptides act as chemoattractants for

fibroblasts *in vitro* (Postlethwaite, 1978)

Laminin

This is a major constituent of basement membranes and is in contact with a variety of epithelial and mesenchymal cells. Purified laminin has been shown to exhibit disparate biological activities which include cell proliferation, attachment, and chemotaxis (Terranova et al., 1980), inhibition or enhancement of metastasis and angiogenesis (Kleinman et al., 1989), induce collagenase type IV (Timpl et al., 1983), and induce differentiation of neuronal precursor cells and tyrosine hydroxylase enzymes (Raghow, 1994).

Our laboratory has earlier demonstrated, enhanced healing of punch wounds created in the dorsal surface of mice on treatment with interferon and polyinosinic-polycytidylic acid (poly-IC)-an interferon inducer (Bharatiya et al.,1992). Enhanced wound healing was reported on the basis of gross and histological observations as well as enhanced ECM protein deposition which are important for remodeling. In this report, we demonstrate our observation of enhanced wound healing in curcumin-treated rabbits, rats and guinea pigs compared to untreated controls based on gross morhological and histological observation as well as studies on ECM proteins and growth factor induction which have been reported to be important in the healing process.

MATERIALS

Female rats (250-350gm) were purchased from Charles River Laboratories

(Kingston, NY). The animals were used in compliance with the U.S. Public Health Service Policy on human care and use of animals. Adult rabbits and guinea pigs were obtained from NLAC, Central Drug Research Institute (Lucknow, India). The animals were housed in a room with 12-hour light, and 12-hour dark cycle and were fed Purina fox chow and water ad libitum. Curcumin was isolated from *Curcuma longa* rhizomes at Central Drug Research Institute (CDRI) (Lucknow, India).

METHODS

Creation of Wounds

The animals were anesthetized using pentabarbital. Hair on the dorsal side of subjects was shaved and skin was cleaned with 70% alcohol. An 8mm skin biopsy punch (Acuderm Inc., Ft. Lauderdale, FL) was used to create full thickness dorsal cutaneous wounds under aseptic conditions. Four wounds were created, with two on each side, and thereafter, the animals were individually caged.

Treatment

Rats, guinea pigs, and rabbits were divided into two groups-viz., untreated control and curcumin-treated. In guinea pigs and rabbits, curcumin was administered orally everyday (40mg/kg), while in rats, curcumin was administered intraperitoneally 18h prior to the creation of the wounds, on the day of wounding, and day 1, 3 and 5 post-wounding. Rats and guinea pigs were sacrificed on day 3 and day 6 after wounding,

while rabbits were sacrificed on day 4 and day 7 post-wounding.

Histological studies

The wound lesions were photographed at the beginning till the termination of the experiment. The animals were euthanized using pentabarbital. The wounded skin and the adjacent skin were collected and fixed by immersing in POLY/LEM fixative (Polyscience, Warrington, PA) for histological, immunohistochemical, and *in-situ* studies. For histological studies, the wounded tissues were dissected along the longitudinal plane and the biopsies of the tissue specimens were fixed in 10% formalin in phosphate buffer, pH 7.4. The specimens were fixed at 4°C overnight and then paraffin embedded. Microtome cut sections (4μm) of the tissue specimens were mounted on poly L-lysine coated slides (Sigma Diagnostics, St.Louis, MO), and oven-dried at 59°C. For processing, the paraffin-embedded, fixed -tissue sections were de-paraffinized immediately before staining with two 10min incubations in Hemo-D (Fischer Scientific, Pittsburg, PA), two 10min incubations in absolute ethanol, one 10min incubation in 95% ethanol followed by one 10min incubation in 70% ethanol. The slides were rinsed with running water and soaked in PBS pH 7.4 for 15min.

Quantitation of the Lesion Area

The wound lesions on the animals were photographed on day 5-11 post-wounding. The lesions were scanned and quantitated on a Macintosh 11 cx computer, equipped with Mac Image 3 (copyright 1986-1990). After scanning the images in Adobe

photoshop software, the images were analyzed using NIH Image program.

Massons Trichrome staining

The tissue sections were de-paraffinized and stained for collagen with Masson's trichrome stain. The sections were then photographed under a microscope fitted with a camera.

Immunohistochemical Localization

Immunohistochemical analysis was performed using antibody against laminin (rabbit polyclonal (Cappel, Durham, NC), fibronectin (goat anti-rabbit IgG) (Cappel, Durham, NC) EGF and TGF-β1 (turkey anti-human IgG) (Vector Laboratories, Burlingham, CA) using an indirect avidin-biotin-immunoperoxidase technique (Vectastain-ABC Elite kit, Vector Laboratories, Burlingham, CA) as specified by the manufacturer. Briefly, sections placed on poly-L-lysine coated slides were de-paraffinized and hydrated using graded ethanol. Endogenous peroxidase was blocked with hydrogen peroxide in methanol. Non-specific staining was blocked with normal serum, and the sections were incubated with primary antibody goat anti-rabbit FN or rabbit polyclonal anti-LMN or turkey anti-human TGF-β antibody for 10h at 4°C. To ascertain that the reactions of antibodies were specific, sections from each test were incubated with normal serum IgG separately. Slides were washed with 1XPBS. Biotinylated secondary antibody was added for 1h, followed by avidin-biotin-peroxidase complex for 30min. Slides were stained with heamatoxylin/eosin. The slides were

dehydrated and mounted on Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) and viewed under an Olympus light microscope equipped with a camera.

RT-PCR analysis (Reverse transcription-polymerase chain reaction)

Isolation and purification of RNA

On day 3/4 post-wounding, the animals were sacrificed, the wound tissue excised, washed in PBS containing DEPC-treated water and total RNA isolated immediately using RNAzol (Tel-test Inc., Friendswood, TX) as specified by the manufacturer. RNA was further purified by lithium chloride precipitation, and analyzed by electrophoresis on a denaturing 1.5% formaldehyde gel to ensure the quality of the RNA.

Reverse transcrition reaction

The reverse transcrition reaction used here is a modification of the one described by Diamond et al., (1990). In a final volume of 20μl containing 1.5μl of a 10mM mix of all four nucleotide triphosphates (dNTPs) (Boehringer Mannheim, San Diego, CA); 4μl of 5X reverse transcriptase bufffer (250mM Tris-HCl pH 8.3, 375mM KCl, 15mM $MgCl_2$); 2μl of 0.1M DTT; 2 μl of (1mg/ml) total RNA; 2 μl of random primer (0.5U/25μl) (Promega, Madison, WI); and 7μl of RNAse free double distilled water, RNA and primers were added and heated at 70°C for 10 min to anneal the primers, cooled on ice and the

buffer mix was added. Reverse transcriptase (RT) (200U/μl) (Life Technologies, Gaithersberg, MD) was added to the mix and incubated at 37°C for 30 min. The reaction mixture was then heated at 90°C for 10 min to inactivate the RT and chilled quickly on ice.

Polymerase chain reaction (PCR)

The reverse transcribed cDNA was amplified by PCR using sense and anti-sense specific primers. Briefly, to 3μl of RT mix, the following components were added; 6 μl of dNTP mix (100 mM); 10 μl of 10X PCR buffer and 0.5μl of Taq polymerase (5 U/μl); 0.4μl of sense primer (1μg/μl) and 0.4μl of anti-sense primer (1μg/μl) and 79.7μl of distilled water. The samples were incubated in DNA thermocycler (Perkin Elmer Cetus, Gene Amp PCR system 9600) for 25 cycles. To confirm that equal amount of RNA were taken in each PCR reaction in the experiment, primers for the " house keeping gene", glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for comparison.The location of primers for nucleotide sequence amplification of amplified DNA product on Southern blot were bases 1271-1292 (sense), 1510-1491 (antisense) and 1401-1421(probe) for TGF-β1 and 1657- 177 (sense), 2070-2050 (antisense) and probe (1830-1850) for fibronectin, and GAPDH primers were 388-405 (sense), 581-562 (antisense) and 531-549 (probe).

Southern blot analysis of the amplified product

12 μl of the final PCR products from each sample were run on 1%GTG agarose

(Seakem, Rockford, ME), blotted to nytran membrane (Schleicher and Schuell, Keene, NH) and hybridized to ^{32}P (NEN, Boston,MA), end labelled oligo-probes at 42°C for 8 hr and autoradiographed.

In-situ hybridization

Paraffin sections were cut 1μm thick on an ultramicrotome and allowed to adhere overnight at 42°C on silane coated slides (Sigma Diagnostics, St.Louis, MO). The sections were de-paraffinized as mentioned earlier. De-paraffinized sections were first incubated for 30min at 65°C in a solution containing 2XSSC, followed by 0.2M HCl for 20min, and washed twice for 3min in 2XSSC. The sections were incubated for 15min at 37°C with 1ug/ml of proteinase K (Boehringer Mannheim, San Diego, CA) in a solution containing 2mM $CaCl_2$ and 10mM Hcl, pH7.4. The samples were rinsed with PBS containing 2mg/ml glycine, followed by 10min incubation in freshly prepared solution containing 0.25% acetic anhydride and 0.1M triethanolamine, pH 8. The samples were then rinsed with distilled water, dehydrated with successive incubation in 70% and 95% ethanol, and air dried. The samples were heated at 90°C for 6min, cooled on ice, and prehybridized for 16h in solutions containing 10mM DTT, 1mg/ml BSA, 0.6M NaCl, 50% deionized formamide, 10%w/v dextran sulphate, 200ug/ml sonicated salmon sperm DNA, 0.02%w/v Ficoll, 0.02%w/v polyvinylpyrrolidone, 0.5mM EDTA, and 10mM Tris-HCl, pH7.4. The hybridizations were carried out for 50h at 42°C using fresh hybridization solution containing ^{35}S-labelled TGF-β cDNA. The probe was labelled with ^{35}S-dATP(NEN, Boston, MA) by Nick translation to a specific activity of approximately

$3X10^7$cpm/ml of hybridization solution. Following hybridization, the sections were washed in 50% formamide, 2XSSC, 10mMDTT at 50°C, digested with 10ug/ml Rnase A (Sigma Chemical Co., St.Louis, MO), for 30min at 37°C, and then washed for 1h in three changes of a solution of 0.1XSSC, 10mMDTT at 52°C and air dried. The sections were then dipped into photographic emulsion (NTB-2, Eastman Kodak Co., Rochester, NY; diluted 1:2) and exposed for 1 week at 4°C in dessicant containing boxes. The samples were subsequently developed with a photographic developer (D-19, Eastman Kodak) for 30-90sec, rinsed for 30sec with distilled water, and fixed for 5min (Kodak Fixer, Eastman Kodak, Rochester, NY). Sections were subsequently stained with eosin. The specimens were finally dehydrated, mounted with permount (Fisher Scientific, Fairlawn, NJ) and viewed under Zeiss microscope.

RESULTS

Gross observations

Wound punches were made on the dorsal side of guinea pigs, rats and rabbits. The contraction of the wound is considered to be an index of wound healing in animals and humans. The closure of the wound on treatment with/without curcumin was observed visually from day of wounding till almost complete wound closure. Visual observations of the punch wounds in guinea pigs were observed daily till almost complete wound closure. Pictures were taken from 5-11 day post-wounding. It is evident from the picture (Fig.15) that enhanced wound closure occurs in curcumin-treated

animals, as compared to untreated controls. Quantitation of the lesion areas in guinea pigs as determined by densitometric analysis using NIH 1.30u program showed almost complete healing in curcumin-treated guinea pigs as compared to untreated controls (Fig.16).

Histological studies

Multiple cross sections of hematoxylin and eosin stained sections of curcumin-treated and untreated biopsy punch tissues of rats, rabbits and guinea pigs were examined for epithelial regeneration and infiltration of different cells into the wound bed. In 3 day rat wound punches, curcumin-treated sections showed a moderately thick crust covering the exposed dermis (Fig.17B). The crust/scab was composed of degenerating cells (primarily neutrophils and some macrophages), keratin, and mild to moderate number of infiltrating viable neutrophils. Occasionally, proliferating fibroblasts were observed adjacent to the areas where the epithelium has been interrupted. However in untreated controls, the epithelium was not intact and the epithelial cells adjacent to the area of loss did not exhibit hyperplasia. Proliferating fibroblasts as well as neovascularization was not seen in the dermis although, there were moderate to marked number of inflammatory cells infiltrating throughout the dermis under the area of epithelial loss (Fig.17A).

Tissue sections from day 6 post-wound area of untreated controls appeared to be essentially the same as that of day 3 treated wound tissues (Fig.17C). There was a moderate to thick crust covering the dermis, and mild neovascularization and

granulation tissue formation was observed. Curcumin-treated wound sections on day 6 post-wounding however, showed almost complete re-epithelialization along with newly formed epidermis (Fig.17D).

In rabbit tissues, curcumin-treated sections showed partially intact epithelium, exhibiting a mild to moderate focally extensive hyperplasia adjacent to the area of epithelial interruption on day 7 post- wounding as compared to day 7 of control (Fig.18). While occasional proliferating fibroblasts were observed within the superficial dermis of curcumin-treated sections, there were no proliferating fibroblasts observed within the dermis in untreated controls. While the dermis area beneath the epithelial loss showed mild neovascularization and infiltration of mild to moderate number of inflammatory cells in curcumin-treated wounded tissues, there was no neovascularization in the case of untreated controls.

Fig.15: Effect of curcumin on wound contraction in guinea pigs: Punch wounds were made on the dorsal side of guinea pigs as described under Methods. Curcumin 40mg/Kg body weight was administered orally everyday, and wound closure was observed visually till almost complete wound closure occured in treated animals. Top panel: Untreated control; Lower panel: Curcumin-treated. Left to right: 1) 5 day; 2) 7 day; 3) 8 day; 4) 9 day; 5) 10 day; and 6) 11 day.

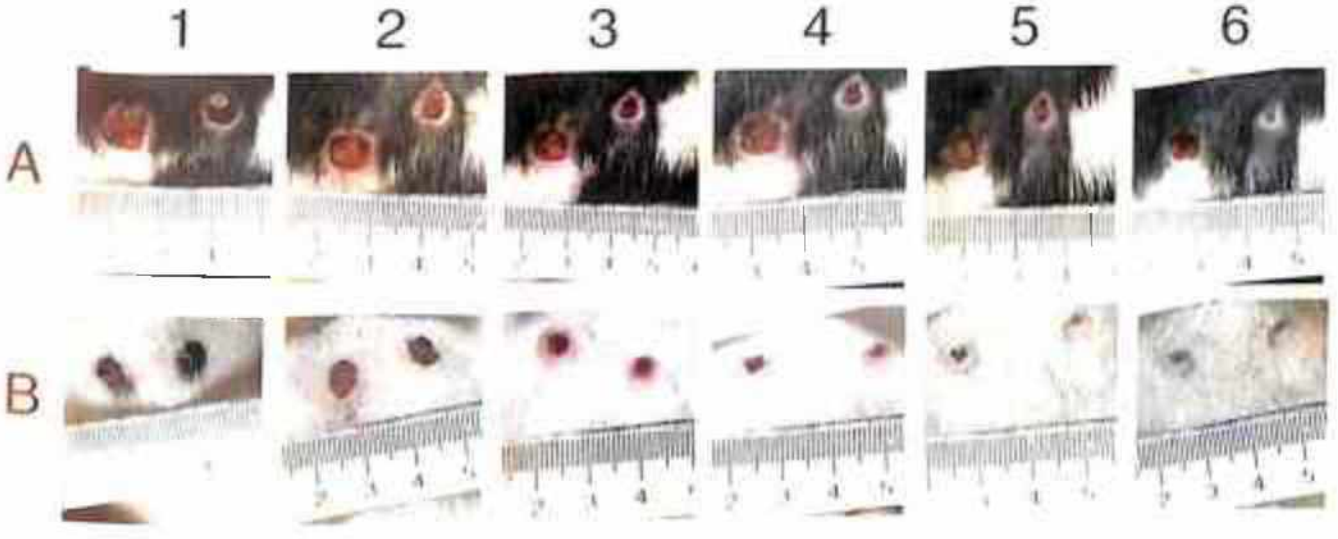

1
2
3
4
5
6
A
B

Fig.16: Quantitation of the wound contraction in guinea pigs: Pictures of the wound area in animals shown in Fig.15 were scanned in Adobe photoshop and the area was quantitated as described under Methods.

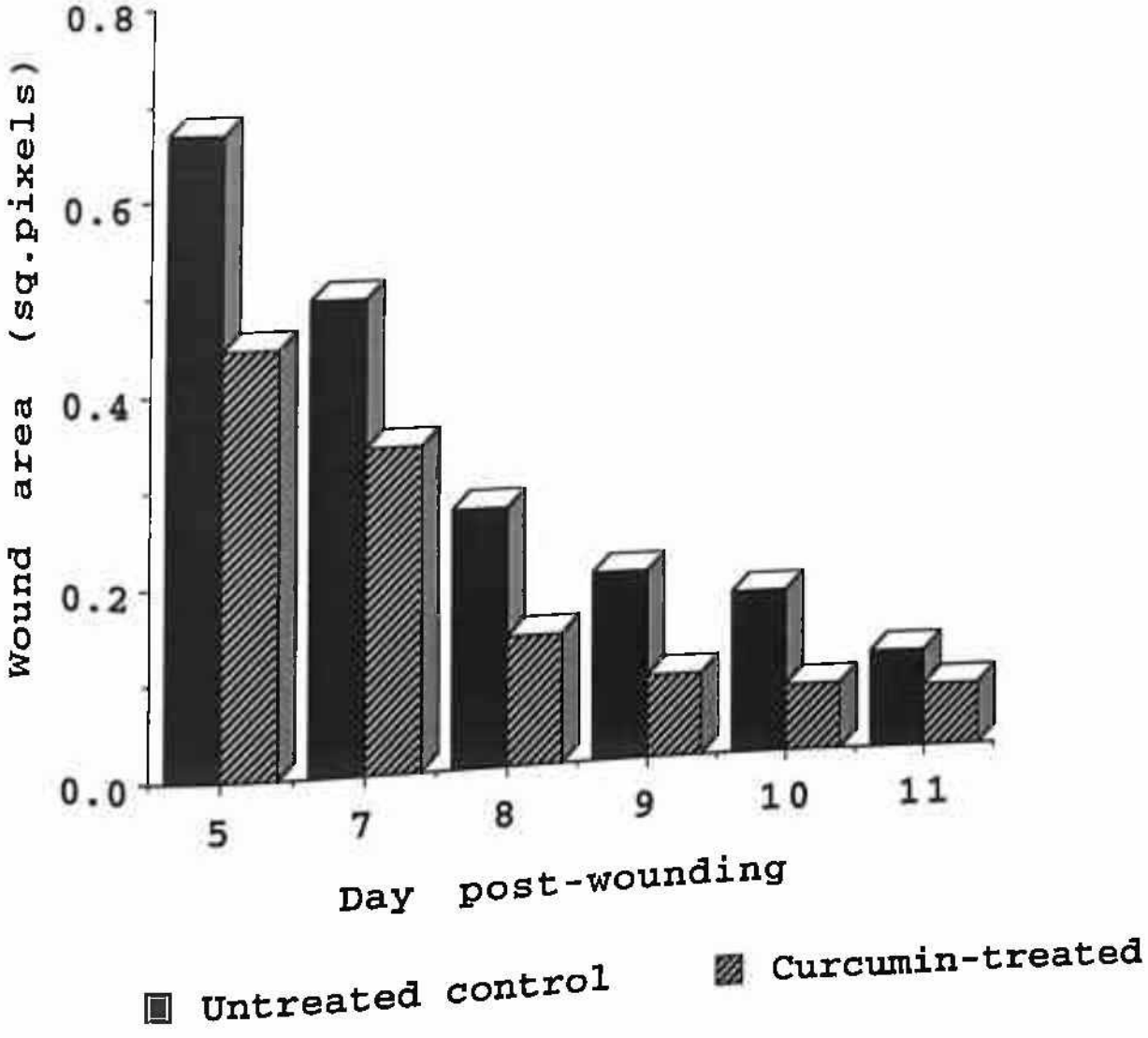

0.8
0.6
0.4
0.2
0.0
Wound area (sq.pixels)
5
7
8
9
10
11
Day post-wounding
Untreated control
Curcumin-treated

Fig.17: Hematoxylin/eosin staining of rat tissues: A) 3 day untreated wound (10X); shows epithelial loss, focally extensive area of cell hyperplasia adjacent to the area of epithelial loss and loose crust over the area of epithelial loss. B) 3 day curcumin-treated wound (10X); Hyperlastic epithelium adjacent to the wound. Migrating epithelium covers the epithelial loss. Area under the dermis is diffusely infiltrated with moderate number of fibroblasts and macrophages. Multiple areas within the dermis exhibit mild to moderate neovascularization; C) 6 day untreated wound (10X); is essentially the same as 3 day curcumin-treated tissues. Sections show epithelial regeneration. Thin focally extensive layer of infiltrating neutrophils/ esionophils just distal to the crust formation. Dermal collagen fibres are loosely arranged; D) 6 day treated wound: Complete re-epithelization with 2-3 layer thick epidermis is formed. The granulation tissue is filled with fibroblasts and macrophages. Exhibits multiple areas of neovascularization. Glandular tissue is fully developed.

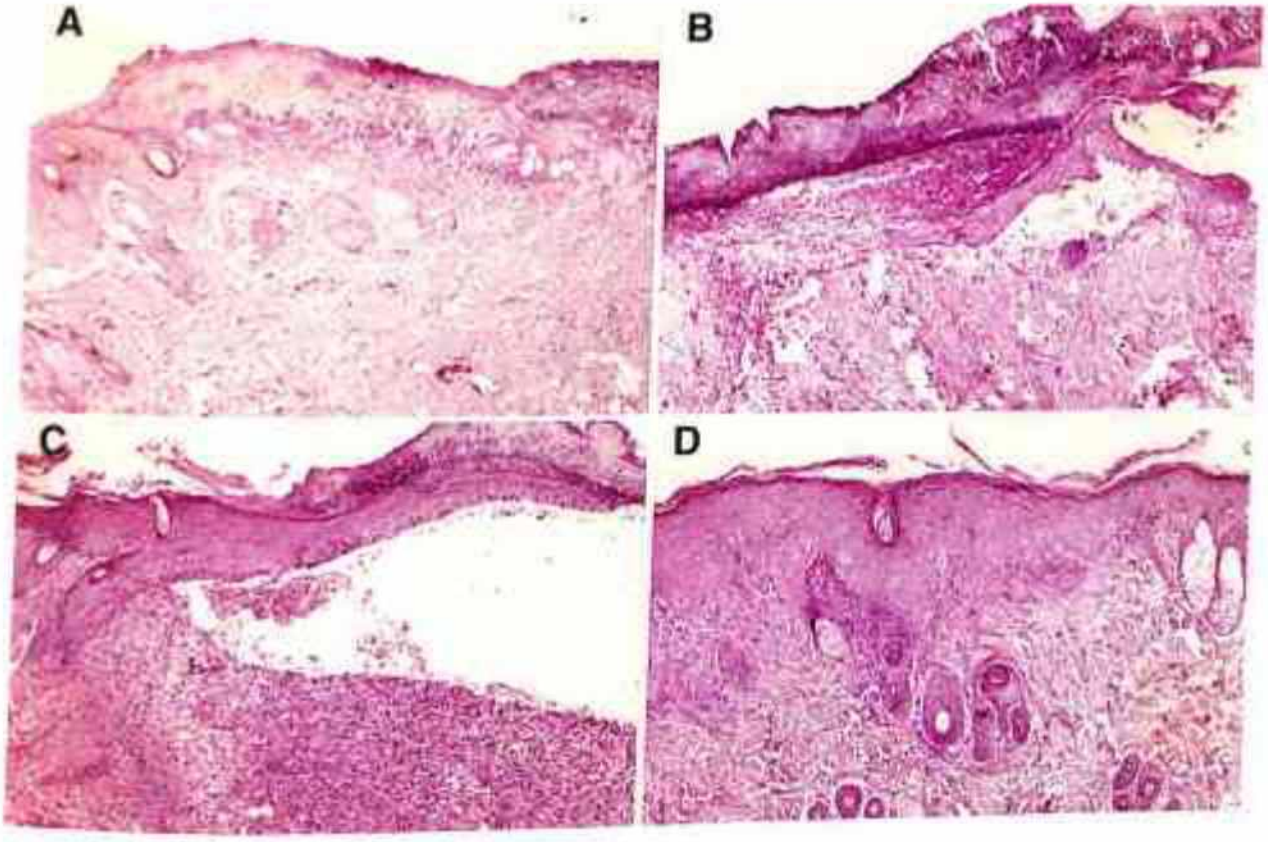
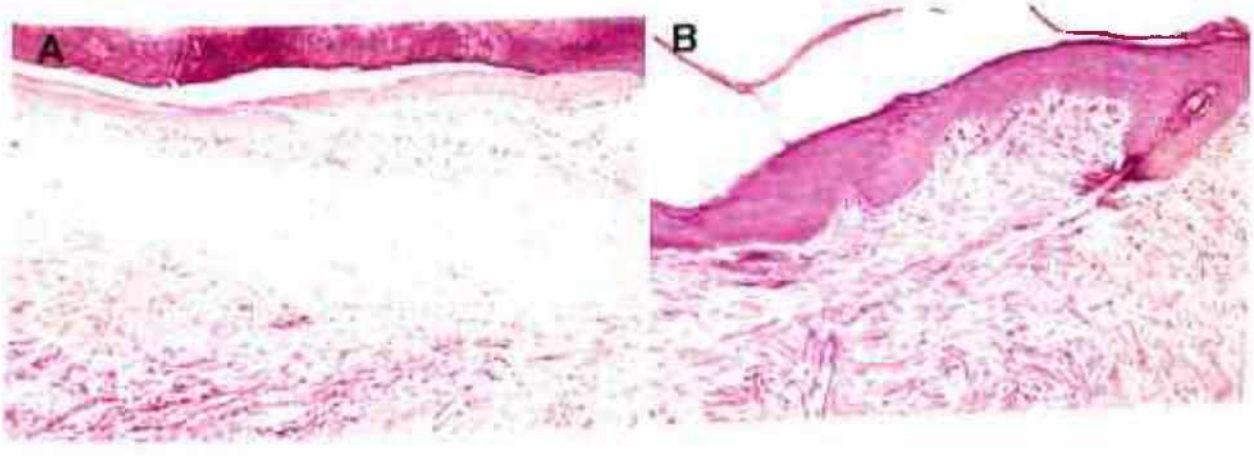

A
B
C
D

Fig.18: Hematoxylin/eosin staining of rabbit tissues: A) Day 7 untreated wounds (10X); B) Day 7 curcumin-treated wounds (10x). Sections from treated/untreated wounds were stained with hemotoxylin/eosin.Treated sections demonstrates complete re-epithelialization and deposition of collagen fibres in the matrix. Untreated sections show a thick scab and partial re-epithelialization. The matrix is filled with migrated inflammatory cells. Moderate neovascularization is seen in the wound bed.


A
B

Effect of curcumin on collagen synthesis by Masson's Trichrome staining

Collagen staining of rabbit tissues with Masson's tricrome stain demonstrated higher collagen content in curcumin-treated tissue sections as compared to untreated controls. Collagen depostion was observed just below the epidermal layer in treated wound sections (Fig.19). On day 6 post-wounding, however, there was no significant difference between treated and untreated controls. Similar results were observed in guinea pigs (Data not shown).

Effect of curcumin on the expression of fibronectin and laminin by immunohistochemistry

ECM proteins were localized in curcumin-treated and untreated wound sections. As compared to a faint staining of fibronectin and laminin in untreated wounds (Fig.20A), fibronectin, however, was found predominantly in the epidermis and granulation tissue of curcumin-treated wounds in guinea pigs while migrating epithelium and the vessels in the dermis show maximum staining for laminin in curcumin-treated wound sections (Fig.20B). Curcumin-treated wounds consistently demonstrated a greater influx of macrophages and fibroblasts into the dermis and stained positive for laminin and fibronectin.

Effect of curcumin on the mRNA transcripts of fibronectin

As immunohistochemical analysis demonstrated an increase in fibronectin expression, we wanted to determine the effect of curcumin on the biosynthesis of fibronectin in the wound area. Using RT-PCR analysis, we observed an increase in the mRNA expression of fibronectin in curcumin-treated wounds (Fig.21). Equal expression of GAPDH inicates that equal RNA was taken for RT-PCR analysis.

Effect of curcumin on EGF expression

Sections of treated/untreated rabbit wounds were stained for EGF using anti-EGF antibody. While in 3 day curcumin-treated sections the staining for EGF was more intense as compared to untreated control, 6 day sections of both treated as well as untreated sections demonstrated almost same intensity of staining (Fig.22). In 3 day curcumin-treated sections the wound-bed appeared to be rich with EGF stained inflammatory cells. However, in 3 day untreated control the absence of this staining was significant.

Fig 19: Masson's Trichrome staining for Collagen in rats: A) 3 day untreated; B) 3 day curcumin-treated; C) 6 day untreated; D) 6 day curcumin-treated wound sections. Sections were de-paraffinized and stained with Masson's trichrome staining for collagen.

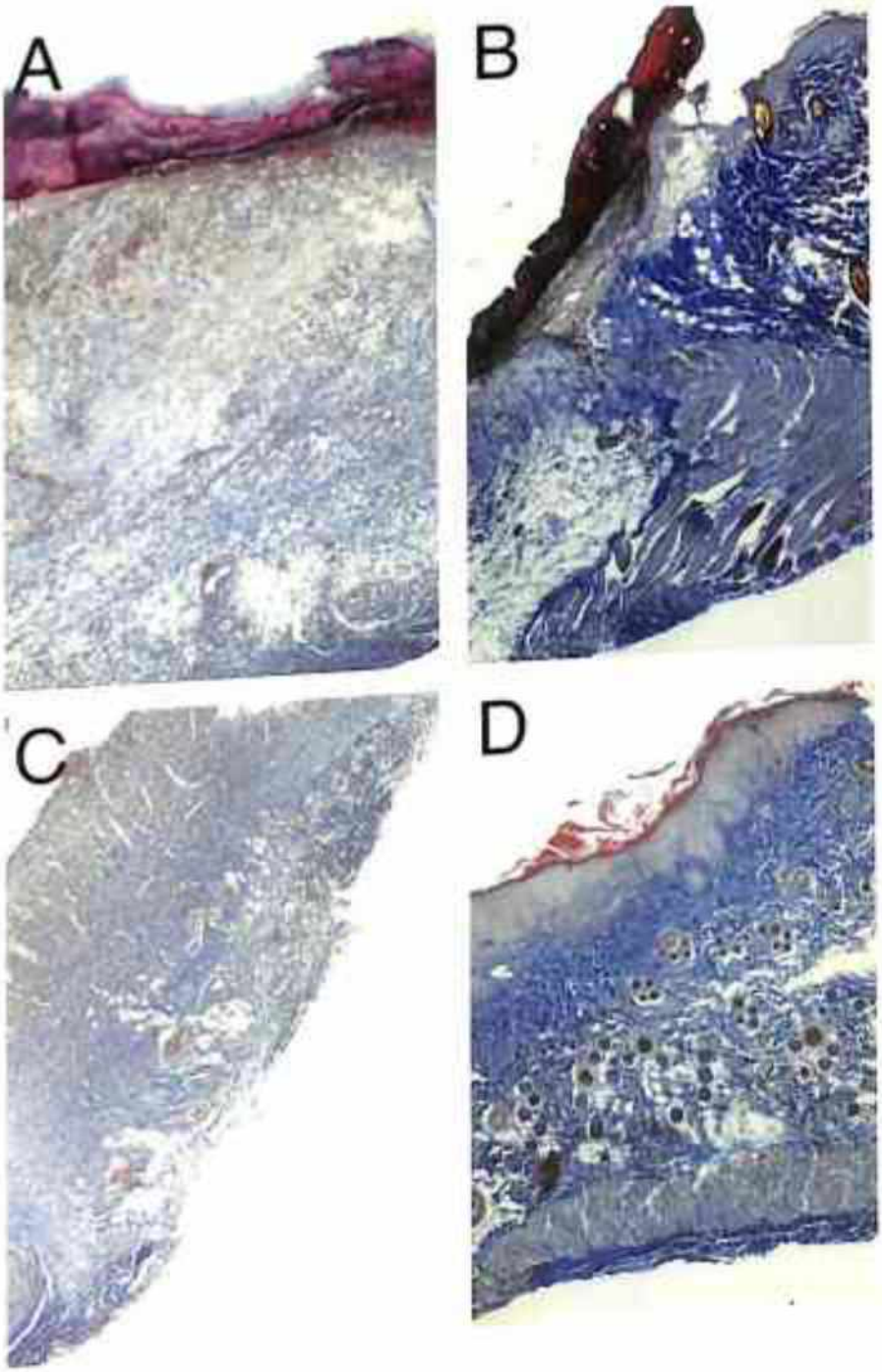

A
B
C
D

Fig.20. Effect of curcumin on laminin and fibronectin expression in guinea pig wounds by immunohistochemistry: Top panel: Curcumin-treated/untreated wound sections were deparaffinized and treated with goat anti-laminin antibody as mentioned under Methods. Bottom panel: Treated/untreated sections were treated with rabbit anti-fibronectin antibody and the expression of fibronectin determined using ABC elite kit (Vector laboratories) as mentioned under Methods A) 3 day untreated guinea pigs; B) 3 day curcumin-treated guinea pigs.

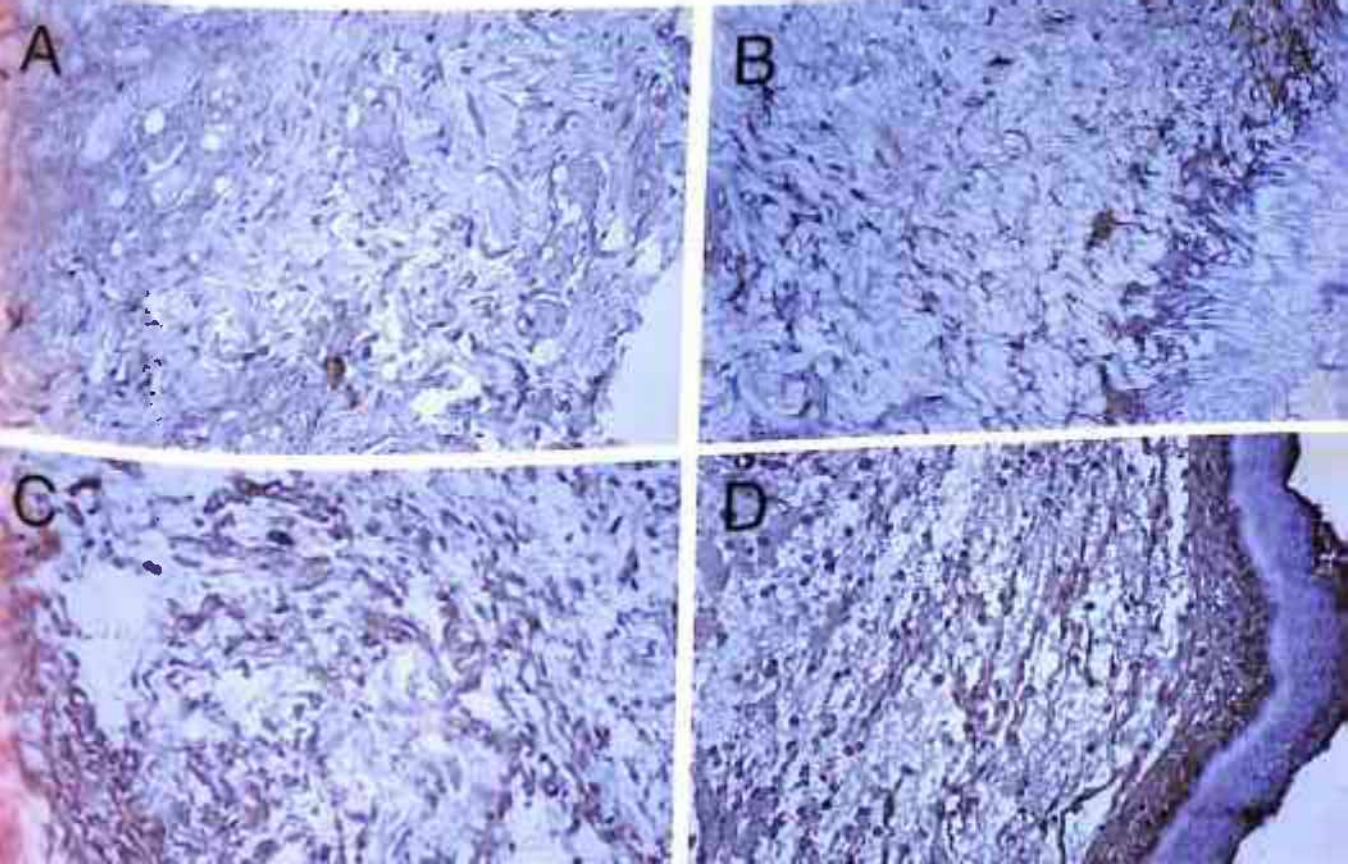

A
B
C
D

Fig.21: Effect of curcumin on fibronectin mRNA transcripts. A) Untreated control; B) Curcumin-treated. Rat wound area on day 6 post-wounding was separated and RNA prepared using RNAzol (Life Technologies, Gaithersburg, MD) as specified by the manufacturer. RNA was reverse transcribed and amplified as mentioned under Methods using specific primers for fibronectin and GAPDH. The amplified products were separated on 1% agarose, transferred to a nylon membrane and hybridized with fibronectin and GAPDH oilgo probes. Equal GAPDH indicates that equal RNA was used at the time of reverse transcription.

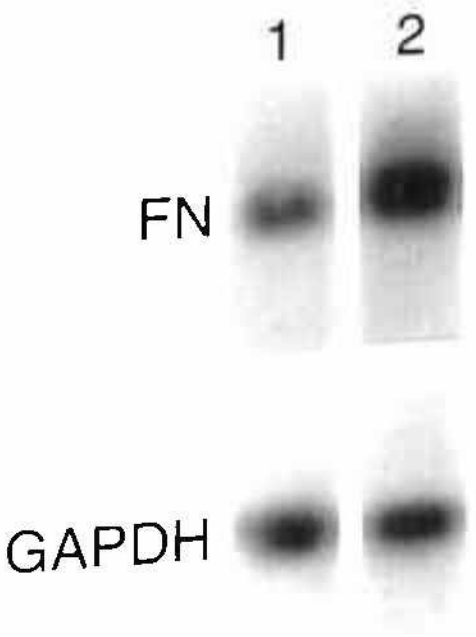
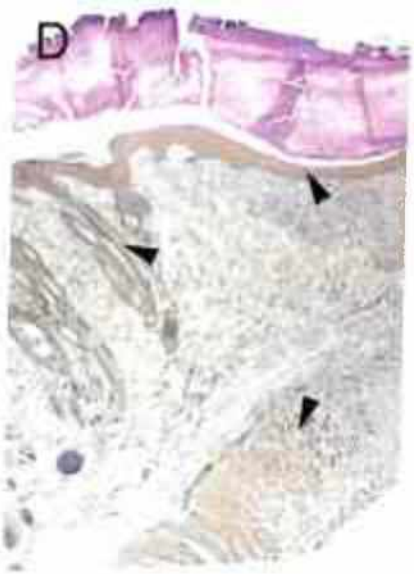

1
2
FN
GAPDH

Fig.22: Effect of curcumin on EGF expression by immunohistochemistry: A) 3 day untreated; B) 3 day curcumin-treated; C) 6 day untreated; D) 6 day curcumin-treated rabbit wound sections. Sections were incubated with anti-EGF antibody and color developed as mentioned under Methods.


B


D

Effect of curcumin on TGF-β1 by immunohistochemical localization

TGF-β1 was localized in curcumin-treated and untreated wound sections. A faint staining of TGF-β1 was seen in untreated wounds of rats and rabbits (Fig.23 and 24). However, TGF-β1 was found to be highly expressed in the epidermis and granulation tissue of curcumin-treated wounds. Migrating epithelium over the lost tissue area, and hyperplastic epithelium adjacent to the migrating epithelium showed maximum staining for TGF-β1 in rabbits. Curcumin-treated wounds consistently demonstrated a greater influx of fibroblasts, which stained positive for TGF-β1. To demonstrate whether the infiltrating cells were fibroblasts, the sections were stained with smooth muscle alpha actin. As is evident from Fig.25, most of the infiltrating cells in the dermis were positive for actin staining, indicating that the migratory cells in the wound bed of curcumin treated wounds were fibroblasts.

Effect of curcumin on the mRNA transcripts of TGF–β1

Immunohistochemical localization showed an increase in the levels of TGF–β1 in the wounded tissues of curcumin-treated animals. Whether curcumin treatment enhanced the biosynthesis of TGF-β1 was examined using *in- situ* hybridization and RT-PCR. The amplification of TGF–β1 by PCR and hybridization with the oligonucleotide probe, showed that there was an increase in the transcripts of TGF-β1 in curcumin treated wounds compared to untreated wound (Fig.26). The mRNA transcripts of

GAPDH show that an equal quantities of RNA was taken for the amplification of TGF-β1.

In tissue biopsy studies, PCR amplification may not provide an exact picture to study the regulation of gene transcription in tissues, because a large volume of the tissue which is not involved in the regulation of the gene of interest may contribute o the RNA, thereby interfering with the actual amount of mRNA transcripts, suggesting that PCR amplification may not be ideal procedure for comparision of genes transcripts in tissues. Therefore, we examined the expression of TGF-β1 in untreated and curcumin-treated wounds by *in-situ* hybridization. The grains demonstrates the level of mRNA transcripts. The presence of grains in the vector control wound tissue was almost negligible, indicating the specificity of the procedure (Fig.27A). However, few grains were observed in the wounded skin when the tissue was hybridized with the cDNA of TGF-β1, indicating the presence of TGF-β1 (Fig.27B).The presence of a large number of grains in curcumin treated wounds showed an increase in the expression of TGF-β1 as compared to untreated wounds (Fig.27C)

Fig.23: Effect of curcumin on TGF-β1 expression by immunohistochemistry: A) 3 day untreated; B) 3 day curcumin-treated; C) 6 day untreated; D) 6 day curcumin-treated rat wounds sections. Treated/untreated wound sections were deparaffinized and treated with anti-TGFβ1 antibody. Turkey anti-human IgG (Vector laboratories, Birmingham, CA) was used as secondary antibody and color was developed as mentioned under Methods.

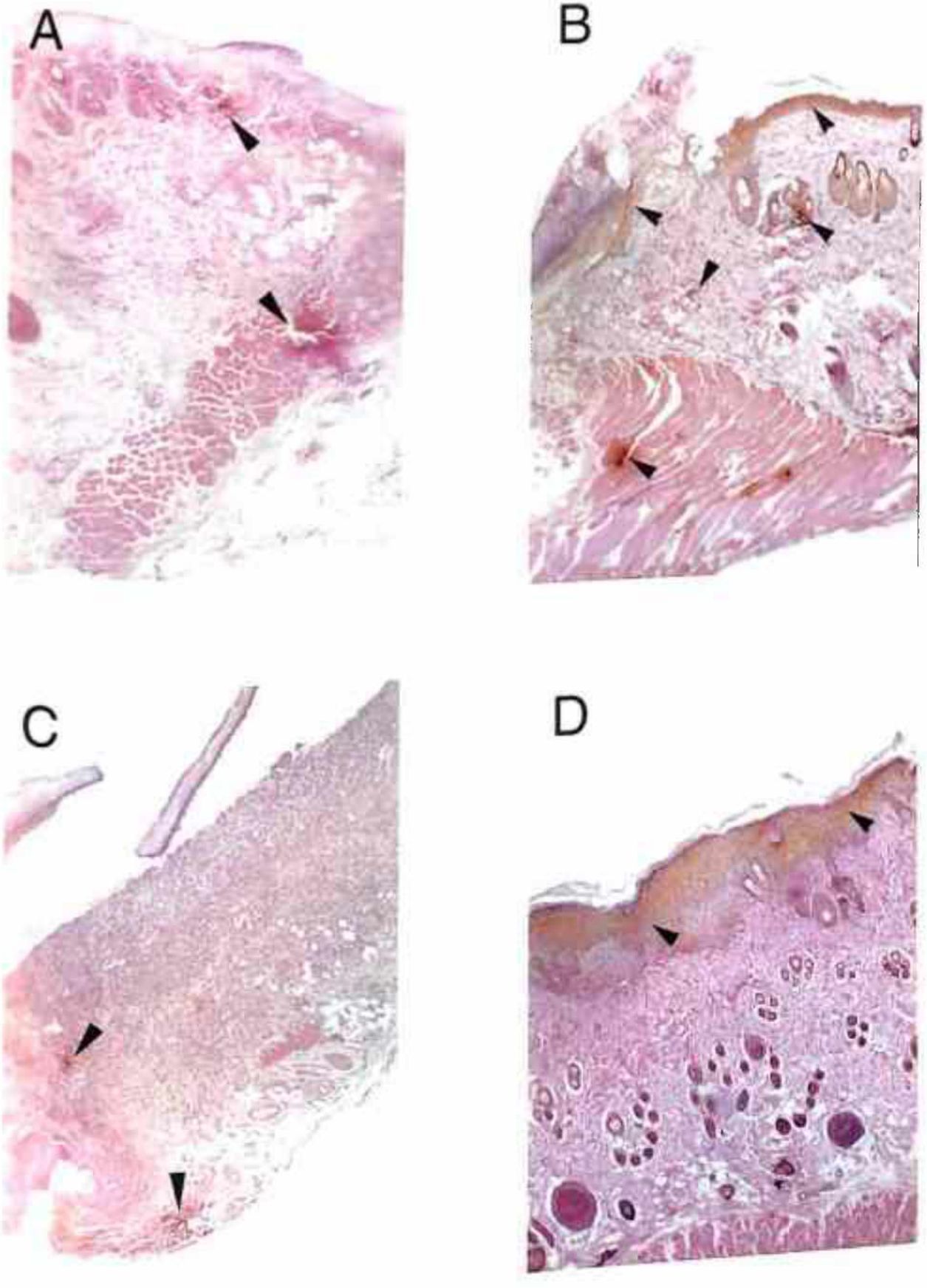
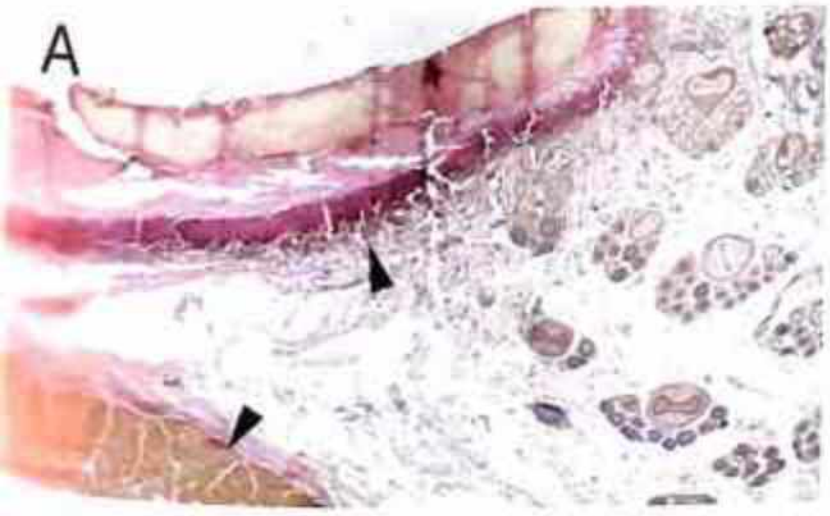
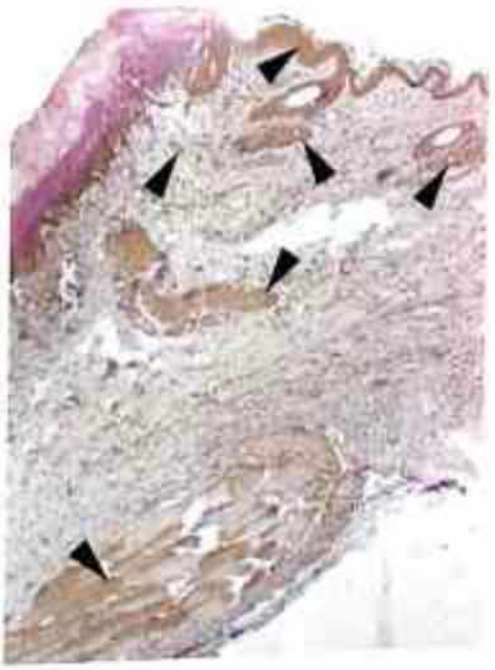
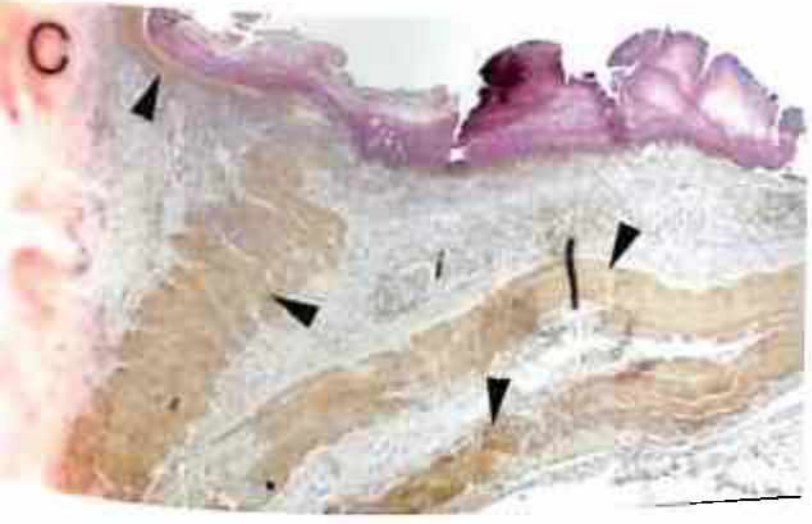
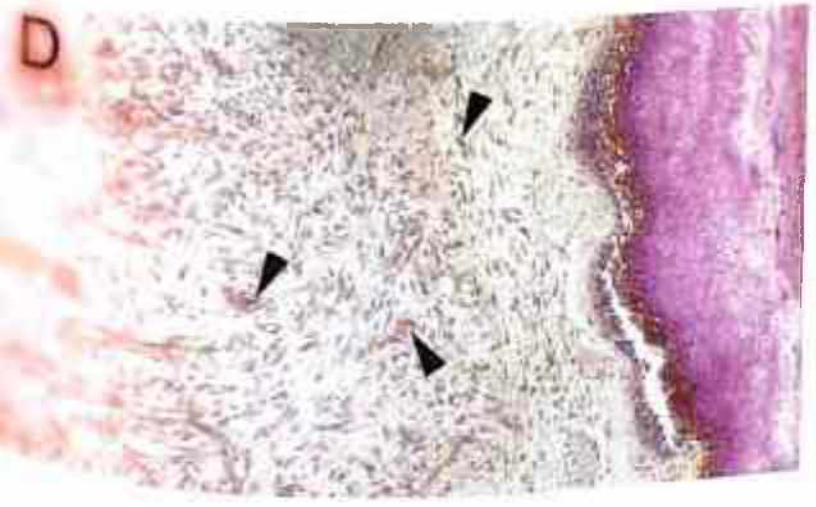

A
B
C
D

Fig.24: Immunohistochemical localization of TGF-β1 in rabbits: A) 4 day untreated; B) 4 day curcumin-treated; C) 7 day untreated-; D) 7 day curcumin-treated wounds. E) Higher magnification of 4 day curcumin-treated wound representing the granulation tissue. Proliferating cells in the matrix are showing staining for TGF-β1.


A


B


C


D

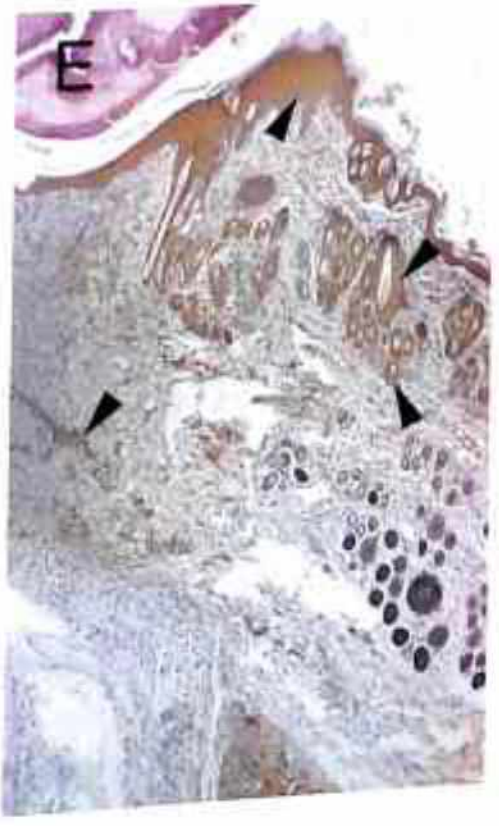

E

Fig.25: Smooth muscle α -actin staining in rabbit wound: Treated/untreated wound sections were stained with anti-α actin antibody. Infiltrating cells in the wound bed after 6 day of wounding and curcumin treatment show positive staining for actin, indicating the migrating myofibroblasts.

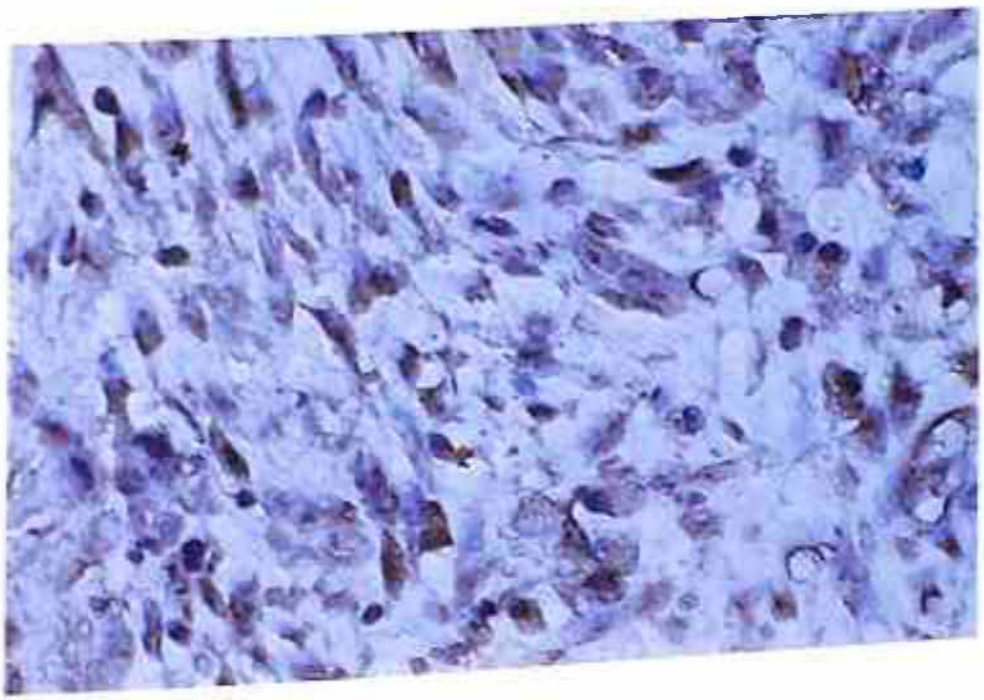


Fig.26: Effect of curcumin on TGF-β1 mRNA in rat wounds: Curcumin-treated/untreated wounds on 3 days post-wounding were separated and RNA made using RNAzol (Life Technologies, Gaithersburg, MD). Equal RNA was reverse-transcribed and amplified using specific primers for TGF-β1 and GAPDH respectively, as mentioned under Methods. The amplified product was separated on 1% agarose gel and transferred to nylon membrane and probed with TGF-β1 and GAPDH oligo probes. Lanes: A. Untreated control; B. Curcumin-treated rats.

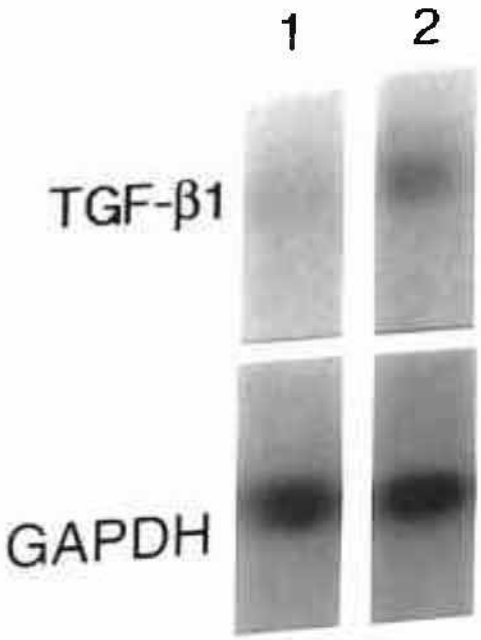
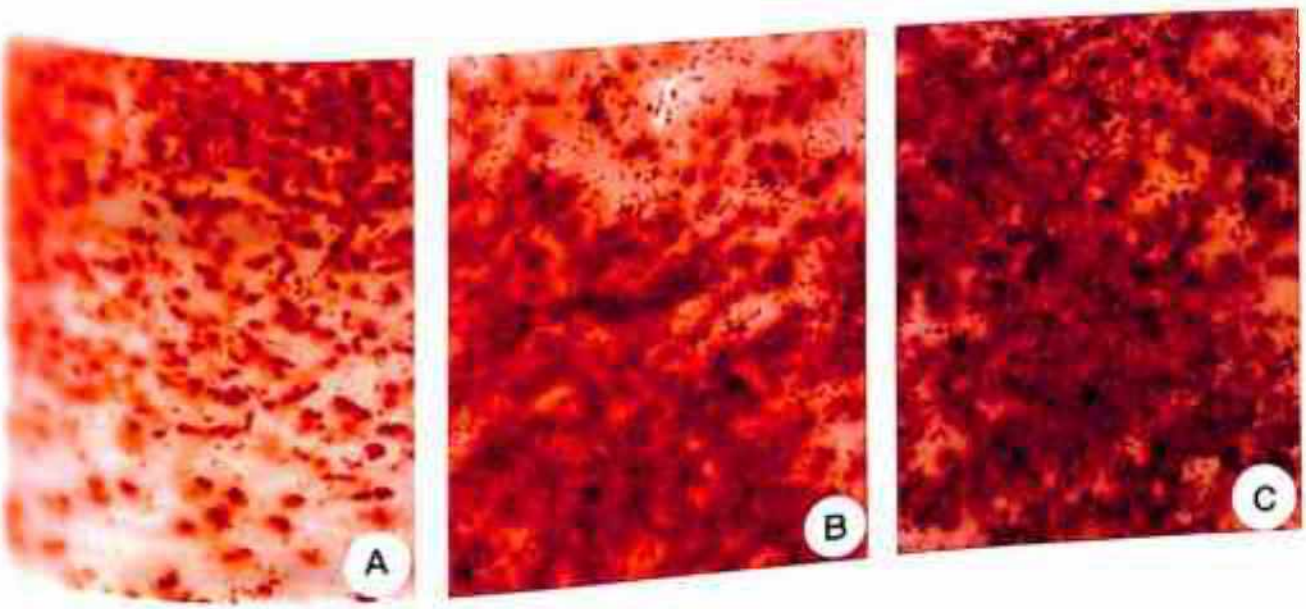

1
2
TGF-β1
GAPDH

Fig.27: *In-situ* hybridization for TGF-β1 in rat wounds: A) Wound section hybridized with plasmid vector PBR322; B) Untreated wound; C) Curcumin-treated wounds. Sections fixed on sialinated slides were processed and hybridized with ^{35}S-radiolabelled TGF-β1 c-DNA, which had been excised from PBR 322 vector, as mentioned under methods. Black grains indicate the degree of mRNA expression of TGF-β1. Lack of grains on hybridization with plasmid from which c-DNA of TGF-β1 was excised indicates the degree of specificity of the probe.


A
B
C

DISCUSSION

Several studies have been carried out to investigate the role of growth factors/cytokines as possible agents to enhance the healing process. Growth factors such as PDGF-AA, PDGF-BB, and EGF applied to the wound area showed an increase in the thickness of the granulation tissue in guinea pigs (Legrand et al.,1993). Supplementation of the wound chambers in Adriamycin induced healing deficit in rats, with growth factors like TGF-β and EGF resulted in accelerated healing as observed by collagen synthesis and cellular proliferation (Lawrence et al.,1986). A combination of growth factors like EGF, TGF-β, and PDGF resulted in complete reversal of the inhibition of wound repair. Addition of individual factors was not as effective in stimulating repair, thereby indicating a synergistic action between growth factors.

Previous work done in our laboratory demostrated the wound healing potential of IFN and Poly-IC, an interferon inducer (Bharatiya et al., 1992). The authors had demonstrated enhanced healing in IFN and Poly-IC-treated mice using punch wound models. In the present study, the effect of curcumin on wound healing was investigated using similar comprehensive and quantitative methods of analysis. A faster rate of wound closure was observed in curcumin-treated wounds as compared to untreated controls. Data demonstrated that curcumin administration in rats, rabbits, and guinea pigs has a significant effect on the regeneration of connective tissue and the process of re-epithelialization in the standardized wounds. Full-thickness punch model has been used in our studies thus, enabling us to study wound closure due to contraction as one

of the parameters for assessing wound healing. The infiltration and proliferation of inflammatory cells into the wound bed plays an important role for matrix deposition and restoration of tissue integrity at the wound site. The migration of cells such as platelets, macrophages, and fibroblasts results in the release of various growth factors which help in the wound-healing process. Curcumin-treated wound biopsies stained by hemotoxylin and eosin show greater migration of inflammatory cells into the wound bed, which could result in better and faster restoration of tissue integrity. The migration of the inflammatory cells have been reported to release growth factors that are instrumental in the evolution and resolution of inflammatory reactions.

The major function of neutrophils during wound healing is in tissue defence against infection by phagocytosis and intracellular killing of microorganisms. Extracellular release of toxic oxygen metabolites, granule proteolytic enzymes which accompanies phagocytosis potentiates tissue injury and prolong the acute inflammatory phase. Infection of wounds by microorganisms leads to an enhanced response by the neutrophils. During neutrophil migration and phagocytosis, release of oxygen metabolites and proteases occur, which have the potential to injure tissues. Although the tissue defences tend to neutralise these metabolites, the factors that control the load of neutrophils and thus of their potentially injurious contents are considered to be an important part of the balances (Hasslet and Henson, 1988). Lutomski et al., (1974), have demonstrated the anti-microbial property of curcumin. The enhanced wound healing observed in animals treated with curcumin could be aided by the antimicrobial property of curcumin which tend to inhibit microorganisms present at the site of injury, as compared to untreated controls. The absence of microorganisms at the wound site

would reduce the toxic metabolites load released by the activity of neutrophils thereby preventing tissue injury and increasing the rate of healing.

Prostaglandins have been shown to play an important role in producing and prolonging the inflammatory reaction (Linz et al., 1994). These have been implicated as mediators of wound healing and scar formation. Studies evaluating the effect of non-steroidal anti-inflammatory agents (Cycloxygenase and lipoxygenase inhibitors) suggest that in contrast to corticosteroids, these agents do not effect the rate of healing, but have a significant effect upon connective tissue remodeling (Blumenkrantz et al., 1972 ; Castor, 1975). Curcumin, a non-steroidal anti-inflammatory agent is a weak inhibitor of prostaglandin synthesis and lipoxygenase and cylcooxygenase synthesis (Srivastava et al., 1990; Huang et al., 1991). The observed increased rate of healing in curcumin treated wounds could be aided by the increased rate of connective tissue formation.

The importance of cellular proliferation and angiogenesis at the wound site is well known. Although we have reported the inhibition of endothelial cell proliferation as well as inhibition of the angiogenic differentiation of HUVEC under *in vitro* conditions, we have also observed that very low concentrations of curcumin do not inhibit the proliferation or the angiogenic differentiation of the cells. In fact very low concentrations of curcumin appears to slightly enhance tube formation as compared to untreated cells (data not shown). Curcumin when administered orally is very poorly absorbed (Ammon and Wahl, 1991). Thus, the concentration of curcumin or its metabolites reaching the wound site would be low and at this concentration curcumin might not inhibit the proliferation or angiogenesis which occurs normally during wound healing.

Evaluation of cytokines from the dressings in the wound area of patients

demonstrated the presence of growth factors like TGF-β, TGF-α, EGF, and interleukins (Ono et al., 1994). The presence of these cytokines indicate their role in wound healing. Exogenous stimuli are of primary importance as inducers of the endogenous cytokines. Cytokines in turn regulate each other by competition, interaction and mutual induction in a series of lymph cascades and circuits with positive or negative feedback effects. TGF-β1, an inhibitor of keratinocyte and endothelial proliferation *in vitro*, when administered to a wound tends to enhance wound healing by inducing the migration of inflammatory cells which in turn tend to secrete more growth factors including TGF-β1. Shah et al., (1994) have reported that aministration of TGF-β1,2 neutralising antibody reduced cutaneous scarring in adult rodents after administration of TGF-β1. Thus, an accurate definition of the physiological role of a given cytokine is further complicated by the fact that many effects may be indirect and dependent on the induction of other cytokines.

In our studies, we show an increase in the presence of TGF-β1, as demonstrated by immunohistochemical localization, *in-situ* hybridization and RT-PCR amplification studies, in the wounded sections of animals treated with curcumin. An increase in the presence of this growth factor in treated wounds could be, due to the release of the growth factor at the wound site by the large number of inflammatory cells which have migrated into the wound bed in response to the chemotactic effect of curcumin, or due to an increase in the transcription of this growth factor at the wound site by the direct action of curcumin. The greater localization of TGF-β1 in the epidermis and dermis on day 4, after wounding, in curcumin treated sections could lead to an increase in the

migration of inflammatory cells as well as an increase in the granulation tissue formation. TGF-β1 administration at the wound site has been reported to increase the formation of granulation tissue as well as increase the transcription of extracellular matrix proteins like collagen type I and III, fibronectin and decreased stromelysin as evidenced by *in-situ* hybridization (Quaglino et al, 1990). The increased levels of TGF-β1 may be due to its release at the wound site by migrating inflammatory cells as well as an increase in the transcription of it at the wound site. TGF-β1 administration at the wound site has been reported to increase the formation of granulation tissue as well as increase the transcription of extracellular proteins like collagen type I and III, and fibronectin. The enhanced expression of TGF-β1 observed on day 7 after wounding in curcumin treated animals could lead to an increase in the granulation tissue deposition. The presence of TGF-β1 on day 7 in untreated control indicated the progression of wound healing at the normal rate of healing. Curcumin may be enhancing the rate of healing by either inducing the expression of TGF-β1 which might be responsible for the observed increased rate of healing or curcumin or its metabolites could be chemotactic which might then stimulate the migration of the fibroblasts and other inflammatory cells which in turn would lead to the release of growth factors which have been implicated in enhanced healing.

Dose responsive effects of PDGF-BB, PDGF-AA, EGF and bFGF on granulation tissue *in a guinea* pig partial thickness skin excison model shows increase in the thickness of the granulation tissue by all the growth factors. Application of EGF has been demonstrated to hasten epidermal regeneration in skin graft donor sites in humans

increasing the rate of granulation tissue matrix deposition. Therefore, curcumin a relatively non-toxic and inexpensive compound might be the drug of choice for wound repair as it induces the expression of growth factos like TGF-β1 and EGF in the wounds as well as increase the biosynthesis of extracellular matrix which are important in remodeling.

HYPOTHESIS

As demonstrated by several authors, curcumin appears to have a wide range of biological properties. While the entire range of the biological activities of curcumin nor its exact mechanism of action is known, in this report, we have shown curcumin to inhibit the differentiation of endothelial cells into tube-like structures on Matrigel, as well as enhance the rate of wound healing in animal models. To explain this paradoxical phenomena, we postulate that the biological action of curcumin depends upon the effective concentration of curcumin reaching the site of action.

Several reports have indicated curcumin to be a potent anti-tumor agent. From all these reports it has been inferred that the route of curcumin administration varies with the tumor site. Thus, while orally administered curcumin is observed to be very effective in inhibiting carcinogen-induced tumors in forestomach, duodenum, colon and tongue (Huang et al.,1994; Tanaka et al., 1994), curcumin was highly effective when applied topically in skin tumors (Huang et al 1992; Azuine and Bhide, 1992). Tumors can be inhibited by inhibiting angiogenesis. Using an *in vitro* model of angiogenesis, we have observed inhibition of tube formation on Matrigel by curcumin in a dose-dependent manner. Thus, while at very low concentrations of curcumin (0.05-1μM), the tube formation appeared to be slightly greater than untreated control, higher concentrations were found to be inhibitory. Similarly, curcumin at very low concentrations did not inhibit cell proliferation, whereas higher concentrations inhibited proliferation. The anti-tumor activity of curcumin when applied orally for duodenal, forestomach or colon cancers or when applied topically for skin cancers can be

explained on the basis, that due to higher concentrations of curcumin available at the tumor site it can inhibit tumors, by inhibiting angiogenesis due to cell proliferation inhibition or due to protease inhibition.

Application of curcumin topically on cutaneous wounds did not result in significant healing. The visual observation of enhanced healing is only due to the superficial drying of wounds. This effect could be due to the strong anti-bacterial activitiy of curcumin (Lutomski et al., 1974), which tend to prevent infection as compared to untreated wounds which have a greater risk of infection. We have observed curcumin-treated wounds to highly express TGF-β1. Several authors have shown TGF-β1 to be a very good wound healing agent by several authors. Besides its wound healing properties, it has also been reported to inhibit tube formation, inhibit proteases, and inhibit endothelial cell proliferation *in vitro* (Frater-Schroder et al., 1986). However, it has been shown to enhance angiogenesis *in vivo*. Thus, TGF-β appears to behave differently under *in vitro* and *in vivo* conditions (Yang and Moses, 1990). RayChoudhury and D'Amore (1991) have speculated that the *in vivo* angiogenic action of TGF-β is not to directly stimulate endothelial cells but to act indirectly by stimulating the migration of monocytes. Upon differentiation into macrophages, these monocytes secrete known angiogenic agents, like bFGF which may be the stimulators of angiogenesis. As reported by Ammon and Wahl (1991), administration of curcumin orally in our experiments probably results in very low absorption of curcumin from the gastro-intestinal tract and this results in very low concentration of curcumin reaching the wound site. At this low concentration, curcumin may be functioning pleuripotently by serving as a chemoattractant thereby

inducing the activation and migration of platelets, monocytes and fibroblasts which results in the release of a number of growth factors like TGF-β, EGF, PDGF etc. These growth factors through the autocrine and paracrine pathways stimulate the fibroblasts and ingrowing capillaries, stimulate ECM proteins and help in remodeling.

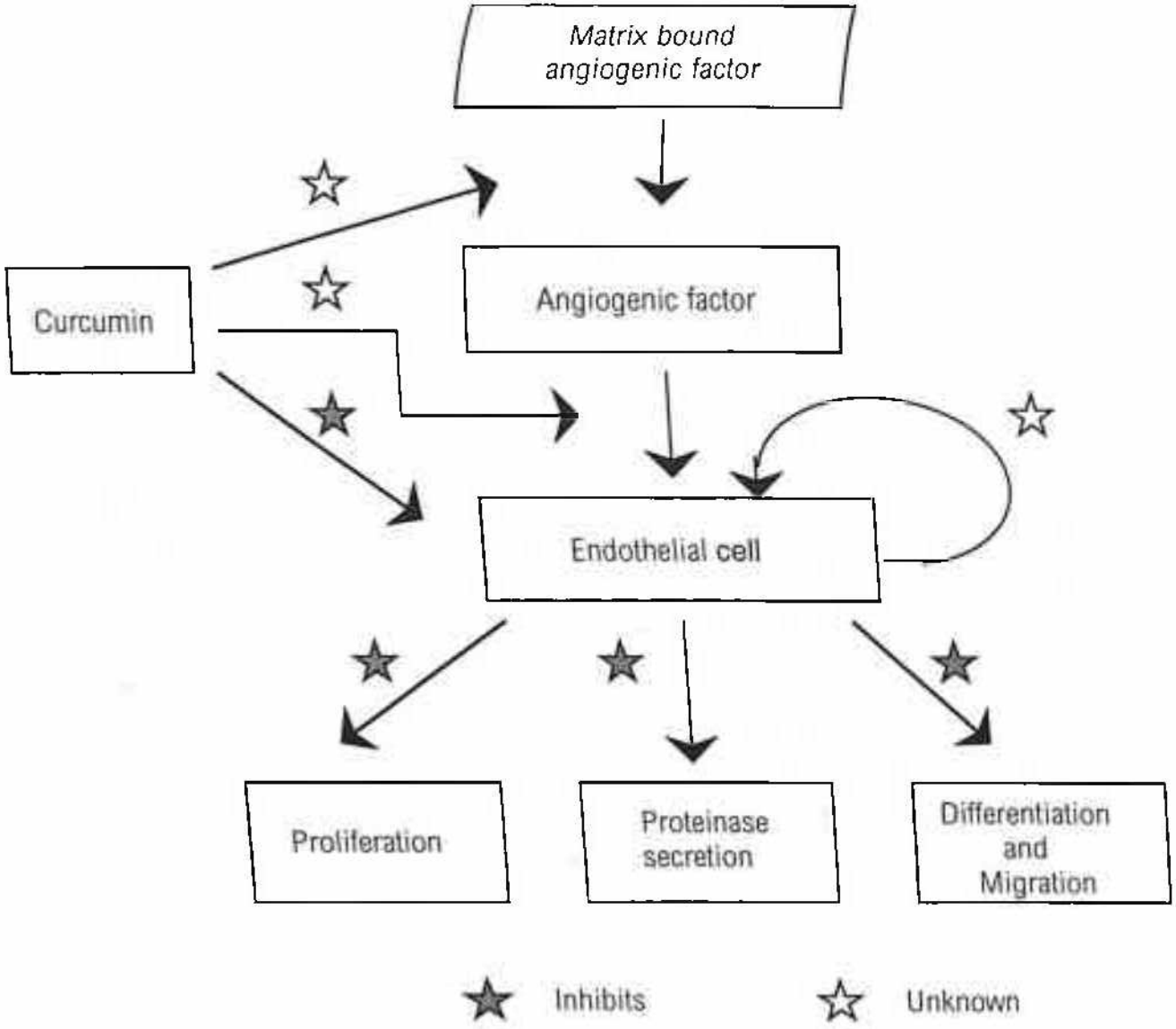
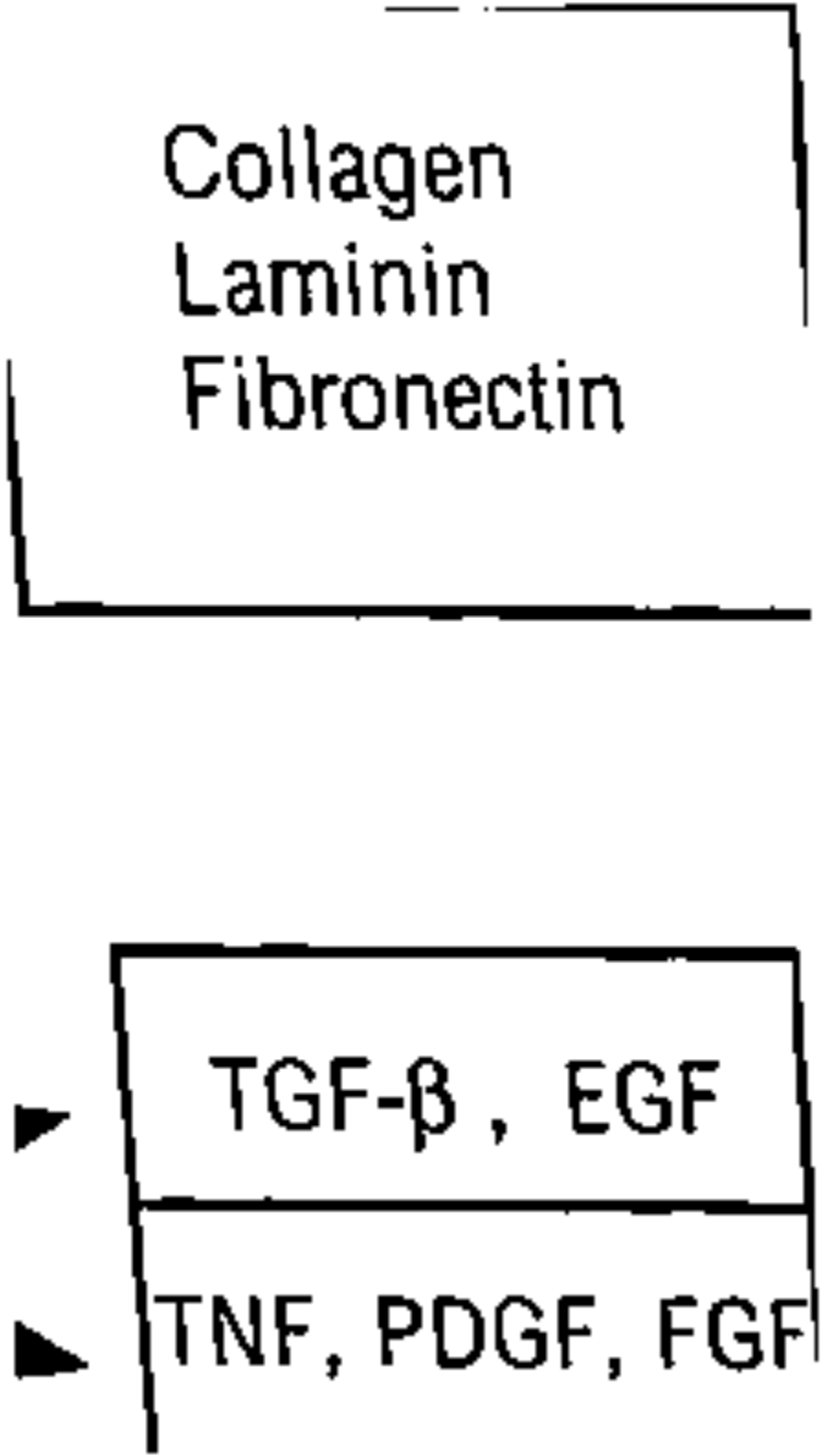


Fig.28:Schematic representation of the inhibition of angiogenesis by curcumin


Collagen
Laminin
Fibronectin
TGF-β , EGF
TNF, PDGF, FGF

BIBILIOGRAPHY

Alliegro MC, Alliegro MA, Cragoe EJ Jr, Glaser BM: Amiloride inhibition of angiogenesis in vitro. J Exp Zool; 267:245-252 (1993).

Ammon HP, Wahl MA: Pharmacology of Curcuma longa. Planta Med; 57:1-7 (1991).

Andrade SP, Fan TP, Lewis GP: Quantitative in vivo studies on angiogenesis in a rat sponge model. Br J Pathol; 68:755-766 (1987).

Ashino-Fuse H, Takano Y, Oikawa T, Shimamura M, Iwaguchi T: Medroxyprogesterone acetate, an anti-cancer and anti -angiogenic steroid, inhibits the plasminogen activator in bovine endothelial cells. Int J Cancer; 44: 859-864 (1989).

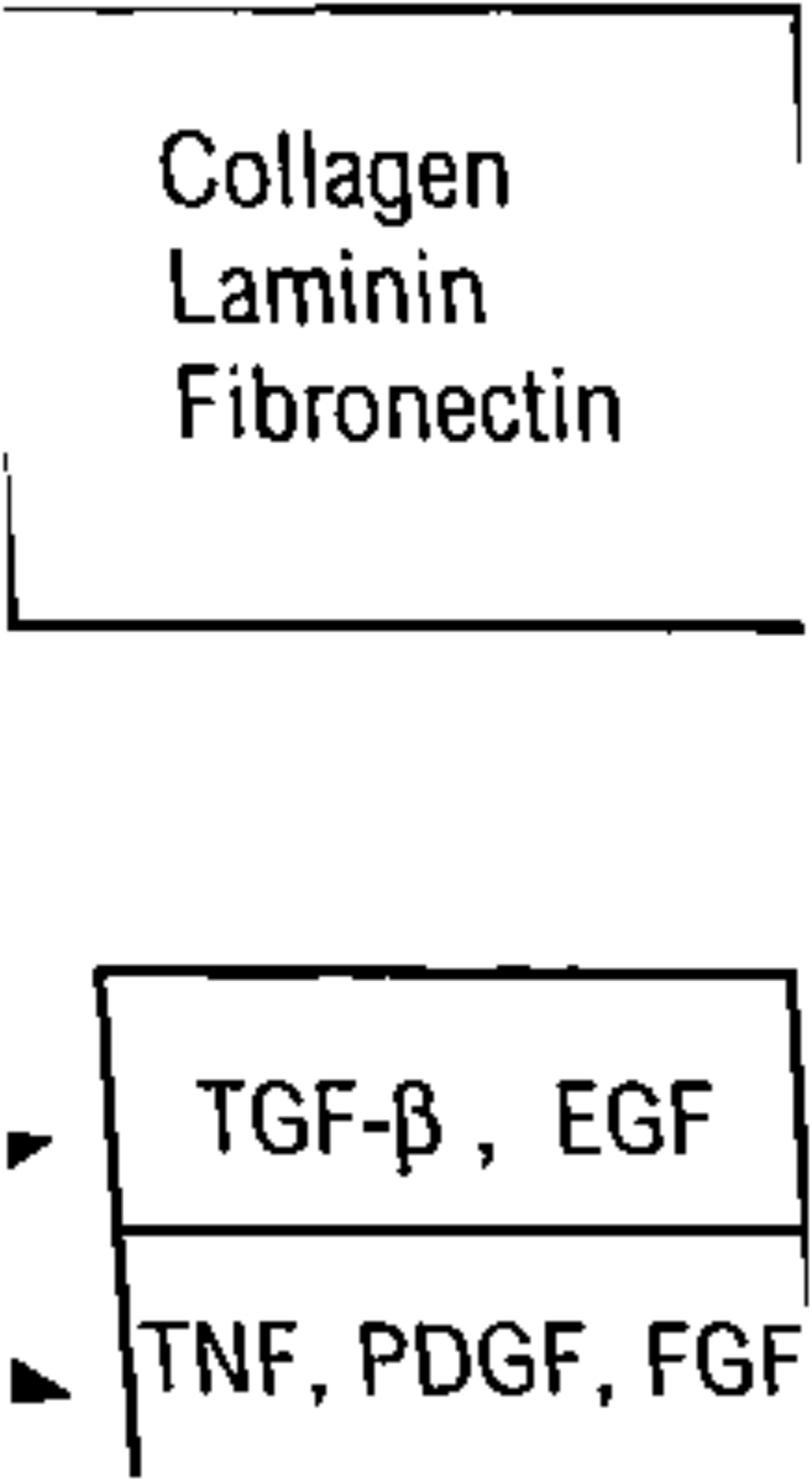
Auerbach W, Auerbach R: Angiogenesis inhibition: A review. Pharmacol Ther; 63:265-311 (1994)

Ausprunk DH, Folkman J: Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc Res; 14:53-65 (1977)

Ausprunk DH, Knighton DR, Folkman J: Differentaition of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev Biol; 38, 237-248 (1974).

Azuine MA, Bhide SV: Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. Nutr Cancer; 17:77-83 (1992).

Bacharach E, Itin A, Keshet E: In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. Proc


Collagen
Laminin
Fibronectin
TGF-β , EGF
TNF, PDGF, FGF

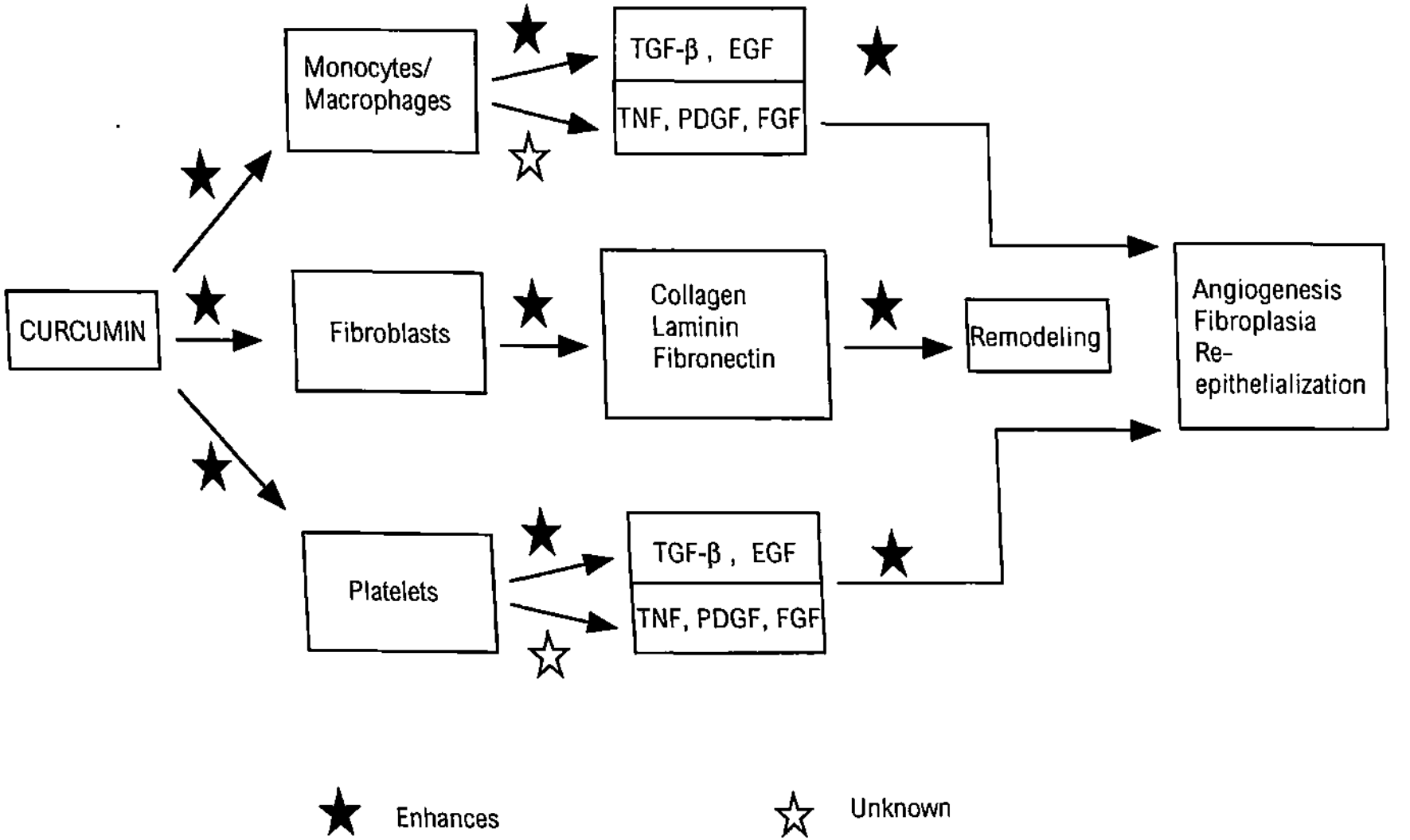


Fig.29: Schematic representation of enhanced wound healing by curcumin

BIBILIOGRAPHY

Alliegro MC, Alliegro MA, Cragoe EJ Jr, Glaser BM: Amiloride inhibition of angiogenesis in vitro. J Exp Zool; 267:245-252 (1993).

Ammon HP, Wahl MA: Pharmacology of Curcuma longa. Planta Med; 57:1-7 (1991).

Andrade SP, Fan TP, Lewis GP: Quantitative in vivo studies on angiogenesis in a rat sponge model. Br J Pathol; 68:755-766 (1987).

Ashino-Fuse H, Takano Y, Oikawa T, Shimamura M, Iwaguchi T: Medroxyprogesterone acetate, an anti-cancer and anti -angiogenic steroid, inhibits the plasminogen activator in bovine endothelial cells. Int J Cancer; 44: 859-864 (1989).

Auerbach W, Auerbach R: Angiogenesis inhibition: A review. Pharmacol Ther; 63:265-311 (1994)

Ausprunk DH, Folkman J: Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc Res; 14:53-65 (1977)

Ausprunk DH, Knighton DR, Folkman J: Differentaition of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev Biol; 38, 237-248 (1974).

Azuine MA, Bhide SV: Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. Nutr Cancer; 17:77-83 (1992).

Bacharach E, Itin A, Keshet E: In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. Proc

Natl Acad Sci USA; 89:10686-10690 (1992).

Bagavandoss P, Kaytes P, Vogelli G, Wells PA, Wilks JW: Recombinant truncated thrombospondin-1 monomer modulates endothelial cell plasminogen activator inhibitor-1 accumulation and proliferation in vitro. Biochem Biophys Res Comm;192:325-332 (1993).

Banda MJ, Knighton DR, Hunt TK, Werb Z: Isolation of a non-mitogenic angiogenesis factor from wound fluid. Proc Natl Acad Sci USA; 79:7773-7775 (1982).

Beutler BA, Cerami A: Cachectin and tumor necrosis factor as two sides of the same biological coin. Nature; 320: 584-588 (1986).

Bharatiya D, Sklarsh JW, Maheshwari RK: Enhanced wound healing in animal models by interferon and interferon inducer. J Cell Physiol; 150:312-319 (1992).

Birkedal-Hansen H, Taylor RE: Detergent-activation of latent collgenase and resolution of its component molecules. Biochem Biophys Res Commun; 107:1173-1178 (1982).

Blumenkrantz N, Sondergaard S: Effect of prostaglandins E1, F1 on biosynthesis of collagen. Nature (New Biol); 239:246-248 (1972).

Bond JS and Butler PE: Intracellular proteases. Ann Rev Biochem; 56: 333-364 (1987).

Brouet I, Ohshima H: Curcumin an anti-tumor promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthetase in activated macrophages. Biochem Biophys Res Commun; 6:533-540 (1995).

Brown GL, Nanney LB, Griffen J: Enhancement of wound healing by topical treatment with epidermal growth factor. N Engl J Med; 321:76-79 (1989).

Brown RL, Breeden MP, Greenhalgh DG: PDGF and TGF-α act synergistically to improve wound healing in the genetically diabatic mouse. J Surg Res; 56:562-570 (1994).

Carey DJ: Control of growth and differentiation of vascular cells by extracellular matrix proteins. Annu Rev Physiol; 53:611-677 (1990).

Castor CW: Connective tissue activation: Evidence supporting a role for prostaglandins and cyclic nucelotide. J Lab Clin Med; 85:392-396 (1975).

Chin JR, Murphy G, Werb Z: Stromelysin a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. J Biol Chem; 260:12367-12376 (1985).

Clark RAF: Biology of dermal wound repair. Dermatologic Clinics; 11:647-666 (1993).

Clark RAF: Basics of cutaneous wound repair. J Dermatol Surg Oncol; 19:693-706 (1993).

Clark RAF: Fibronectin matrix deposition and fibronectin receptor expression in healing and normal skin. J Invest Dermatol; 94:128S-134S (1990).

Clark RAF, Folkvord JM, Hart CE: Platelet isoforms of platelet-derived growth factor stimulate fibroblasts to contract collagen matrices. J Clin Invest: 84:1036-1040 (1989).

Clark RAF: Overview and general considerations of wound repair in: The molecular and cellular biology of wound repair. (Edts) Clark RAF and Henson PM. Plenum Press, New York and London 3-23 (1988).

Cozzolino F, Torcia G, Braunstein N, Butura C, Brett J, Libermann HB, Furie MB,

Joseph SJ, Stern D: Macrovascular and microvascular endothelium during long-term hypoxia: alterations in cell growth, monolayer permeability, and cell surface coagulation properties. J Cell Physiol; 146:8-17 (1991).

Curran T, Franza BR Jr: Fos and Jun: The AP-1 connection. Cell; 55: 395-397 (1988).

Carrico T, Mehrhof A, Cohen I: Biology of wound healing. Surg Clin North Am; 64:721-(1984).

Devereux DF, Thibault L, Boretos J, Brennan MF: The qualitative and quantitative impairment of wound healing by Adriamycin. Cancer; 43:932-938 (1979).

Dethlefsen SM, Shepro D, D'Amore P: Arachidonic acid metabolites in bFGF, PDGF, and serum-stimulated vascular cell growth. Exp Cell Res; 212:262-273 (1994).

Diegelman RF, Cohen IK, Kaplan AM: The role of macrophages in wound repair: A review. Plast Reconstr Surg; 68:107-113 (1981).

Diglio CA, Grammas P, Giacomelli F, Weiner J: Angiogenesis in rat aorta ring explant cultures. Lab Invest; 60: 523-531 (1989).

Dikshit M, Rastogi L, Shukla R, Srimal RC: Prevention of ischemia-induced biochemical changes by curcumin and quinidine in the rat heart. Indian J Med Res; 101:31-35 (1995).

Donatus IA, Sardjoko X, Vermeulen NPE: Cytotoxic and cytoprotective activities of curcumin. Effects on paracetamol induced cytotoxicity, lipid peroxidation and glutathione depletion in rat hepatocytes. Biochem Pharmacol; 39:1869-1875 (1990).

Eaton DL, Scott RW , Baker JB: Purification of human fibroblast urokinase proenzyme and analysis of its regulation by proteases and protease nexin. J Biol Chem; 259:6241-

624 (1984).

Elliott LH, Wilkinson SE, Sedgwick AD, Hill CH, Lawton G, Davis PD, Nixon JS: K252a is a potent and selective inhibitor of phosphorylase kinase. Biochem Biophys Res Commun; 171:148-154 (1990).

Ewing MW, Liu SC, Gnarra JR, Walther MW, Meyers CE, Linehan WM: Effect of Suramin on the mitogeinc responses of the human prostate carcinoma cell line PC-3. Cancer Suppl; 71:1151-1158 (1993).

Fajardo LF, Prionas SD, Kowalski J, Kwan HH: Hyperthermia inhibits angiogenesis. Radiat Res; 114:297-306 (1988).

Folkman J, Klagsburn M: Angiogenic factors. Science; 235:442-447 (1987).

Folkman J: Tumor angiogenesis: therapeutic implications. New Eng J Med; 285:1182-1186 (1971a)

Folkman J, Merler E, Abernathy C, Williams G: Isolation of a tumor factor responsible for angiogenesis; J Exp Med; 133: 275-279 (1971b).

Ford HR, Hoffman RA, Wing EJ, Magee M, McIntyre L, Simmons RL: Characterization of wound cytokines in the sponge matrix model. Arch Surg; 124:1422-1428 (1989).

Frater-Schroder M, Muller G, Birchmeier W, Bohlen P: Transforming growth factor-beta inhibits endothelial cell proliferation. Biochem Biophys Res Commun; 137:295-302 (1986).

Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P: Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Proc

Natl Acad Sci USA; 84: 5277-5281 (1987).

Friesel R, Komoriya A, Maciag T: Inhibition of endothelial cell proliferation by gamma-interferon. J Cell Biol; 104:689-696 (1987).

Gailit J, Welch MP, Clark AF: TGF-b1 stimulates expression of keratinocyte integrins during re-epithelialization of cutaneous wounds. J Invest Dermatol; 103:221-227 (1994).

Garlick JA, Taichman LB: Effect of TGF-β1 on re-epithelialization of human keratinocytes in vitro: an organotypic model. J Invest Dermatol; 103:554-559 (1994).

Gimbrone MA Jr, Cotran R, Leapman S, Folkman J: Tumor growth neovascularization: An experimental model using rabbit cornea. J Natl Canc Inst; 52:413-427 (1974).

Gospodarowitz D, Delgado D, Vlodavsky I: Permissive effect of the extracellular matrix on cell proliferation in vitro. Proc Natl Acad Sci USA; 77:4094-4078 (1980).

Grant ME, Heathcote JG, Orkin RW: Current concepts of basement membrane structure and function. Bio Sci Rep; 1:819-842 (1981).

Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR, Kleinman HK: Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell; 58:933-943 (1989).

Grant DS, Kinsella JL, Fridman R, Auerbach R, Piasecki BA, Yamada Y, Zain M, Kleinman HK: Interaction of endothelial cells with a laminin A chain peptide (SIKVAV) in vitro and induction of angiogenesis behaviour in vivo. J Cell Physiol,153:614-625 (1992).

Gschwendt M, Horn F, Kittstein W, Furstenberger G, Besemfelder E, Marks F: Calcium

and phospholipid-dependent protein kinase activity in mouse epidermis cytosol. Stimulation by complete and incomplete tumor promotors and inhibition by various compounds. Biochem Biophys Res Commun;124:63-68 (1984).

Gospodarowicz D, Bialecki H, Greenburg G: Purification of the fibroblast growth factor activity from bovine brain. J Biol Chem; 253:3736-3743 (1978).

Gross JL, Azizkhan RS, Biswas C, Bruns R, Hseih D, Folkman J: Inhibition of tumor growth, vascularization, and collagenolysis in the rabbit cornea by medroxy progesterone. Proc Natl Acad Sci USA; 78: 1176-1180 (1981).

Gross JL, Moscatelli D, Jaffe EA, Rifkin DB: Plasminogen activator and collagenase production by cultured capillary endothelial cells. J Cell Biol; 95:974-981(1982).

Gross JL, Moscatelli D, Rifkin DB: Increased capillary endothelial cell protease activity in response to angiogenic stimuli in vitro Proc Natl Acad Sci USA; 80: 2623-2627 (1983).

Hammar H: Wound healing. Int J Dermatol; 32:6-15 (1993).

Hanemaaijer R, Koolwijk P, Clercq LL, DeVree JA, Van Hinsberg WM: Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. J Biol Chem; 296:803-809 (1993).

Hanslett C, Henson PM: Resolution of inflammation: In the molecular and cellular biology of wound repair. (Edts) Clark RAF and Henson PM. Plenum Press, New York and London, 185-211 (1988).

Haralabopoulos GS, Grant DS, Kleinman HK, Lelkes PI, Papaioannou SP, Maragoudakis ME: Inhibitors of basement membrane collagen synthesis prevent endothelial cell alignment in matrigel in vitro and angiogenesis in vivo. Lab Invest;

71:575-582 (1994).

Hasty KA, Hibbs MS, Seyer JM: Secreted forms of human neutrophil collagenase. J Biol Chem; 261:5645-5650 (1986)

Herndon DN, Nguyen TT, Gilpin DA: Growth factors: local and systemic. Arch Surg; 128:1227-1233 (1993).

Herron GS, Banda MJ, Clark EJ, Gavrilovic J, Werb Z: Secretion of metalloproteinases by stimulated capillary endothelial cells. II. Expression of collagenases and stromelysin activities is regulated by endogenous inhibitors. J Biol Chem; 261:2814-2818 (1986a).

Herron GS, Werb Z, Dwyer K, Banda MJ: Secretion of metalloproteinases by stimulated capillary endothelial cells. I. Production of procollagenase and prostromelysin exceeds expression of proteolytic activity. J Biol Chem; 261:2810-2813 (1986b).

Hosgood G: Wound healing-the role of platelet-derived growth factor and transforming growth factor beta. Veterinary Surgery; 22:490-495 (1993).

Huang HC, Jan TR, Yen SF:Inhibitory effect of curcumin, an anti-inflammatory agent, on vascular smooth muscle cell proliferation. J Pharmacol; 221:381-384 (1992).

Huang MT, Smart RC, Wong GQ, Conney AH: Inhibitory effect of curcumin, chlorgenic acid, caffeic acid and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoyl phorbol-13-acetate. Cancer Res; 48:5941-5946 (1988).

Huang MT, Lysz T, Ferraro T, Abidi TF, Laskin JD, Conney AH: Inhibitory effects of curcumin on invitro lipoxygenase and cyclooxygenase activities in mouse epidermis. Cancer Res; 51:813-819 (1991).

Huang MT, Wang ZY, Georgiadis CA, Laskin JD, Conney AH: Inhibitory effects of curcumin on tumor initiation by benzo(α)pyrene and 7,12-dimethyl benz(α)anthracene. Carcinogenesis; 13:2183-2186 (1992).

Huang MT, Robertson FM, Lysz T, Ferraro T, Wang ZY, Georgiadis CA, Laskin JD, Conney AH: Inhibitory effects of curcumin on carcinogenesis in mouse epidermis. In Phenolic compounds in food and their effects on health II: Antioxidants and cancer prevention. (Edts) Huang MT, Ho CT, and Lee CY. Washington D.C., American Chemical Society; 339-349 (1992).

Huang MT, Lou YR, Ma W, Newark HL, Reuhl KR, Conney AH: Inhibitory effect of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. Cancer Res; 54: 5841-5847 (1994).

Huang TS, Lee SC, Lin JK: Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblasts cells. Proc Natl Acad Sci USA; 88:5292-5296 (1991).

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamura T, Brem H, Folkman J: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. Nature; 348:555-557(1990).

Hunt TK: Wound fluid: the growth environment: In clinical and experimental approaches to dermal and epidermal repair: normal and chronic wounds. (Edts.) Barbul A, Caldwell MD, Eaglstein WH et al., Wiley-Liss, New York. 233-238 (1991).

Ignotz RA, Massague J: Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem; 261:4337-4345 (1986).

Ito KI, Ryuto M, Ushiro S, Ono M, Sugenoya A, Kuraoka A, Shibata Y, Kuwano M:

Expression of tissue-type plasminogen activator and its inhibitor couples with development of capillary network by human microvascular endothelial cells on matrigel. J Cell Physiol; 162:213-224 (1995).

Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins: Identification by morphological and immunologic criteria. J Clin Invest; 52:2745-2753 (1972).

Johnson MD, Kim HRC, Chesler L, Tsao-Wu G, Bouck N, Polverini PJ: Inhibition of angiogenesis by tissue inhibitor of metalloproteinase. J Cell Physiol;160:194-202 (1994).

Kakar SS, Roy D: Curcumin inhibits TPA induced expression of c-fos, c-jun and c-myc protooncogenes messenger RNAs in mouse skin. Cancer Lett; 87:85-89 (1994).

Kalebic T, Garbisa S, Glaser B, Liotta LA: Basement membrane collagen: degradation by migrating endothelial cells. Science; 221:281-283 (1983).

Kanzler MH, Gorsulowsky DH, Swanson NA: Basic mechanisms in the healing cutaneous wound. J Dermatol Surg Oncol; 12:1156-1164 (1986).

Kerr LD, Miller D, Matrisian LM: TGFβ_1 inhibition of transin/stromelysin gene is mediated through a fos binding sequence. Cell; 61: 267-278 (1990).

Kingsnorth AN, Slavin J: Peptide growth factors and wound healing. Br J Surg; 78:1286-1290 (1990).

Kinsella JL, Grant DS, Weeks BS, Kleinman HK: Protein kinase C regulates endothelial cell tube formation on basement membrane matrix, Matrigel. Exp Cell Res; 199: 56-62 (1992).

Kirsner RS, Eaglstein WH: The wound healing process. Dermatalogic Clinics; 11:629-640 (1993).

Kleiman HK, Cannon FB, Laurie GW, Hassel JR, Aumailley M, Terranova VP, Martin GR, Dabois-Dalcq M: Biological activity of laminin. J Cell Biochem; 27: 317-325 (1985).

Kubota Y, Kleinman HK, Martin GR, Lawley TJ: Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol; 107:1589-1598 (1988).

Kudlow JE, MacLennan DH, Berstein A: Molecular biology, oncogenes, signal transduction and clinical implications: In biology of growth factors: Advances in experimental medicine and Biology. (Edts) Kudlow JE, MacLennan DH, Berstein A et al., Vol.24. New York Plenum Press 238-321 (1987).

Kokha R, Waterhouse P, Yagel S, Lala PK, Overall CM, Nortan G, Denhardt DT: Kokha R, Waterhouse P, Yagel S, Lala PK, Overall CM, Nortan G, Denhardt DT: Antisense RNA-induced reduction in murine TIMP levels confers oncogenecity on Swiss 3T3 cells. Science; 243:948-950 (1989).

Kurkinsen M, Vaheri A, Roberts PJ, Stenman S: Sequential appearance of fibronectin and collagen in experimental granulation tissue. Lab Invest; 43:47-51(1980).

Kuzuya D, Masafumi M, Kinsella JL: Reorganization of endothelial cord like structures on basement membrane complex (Matrigel): Involvement of transforming growth factor β1. J Cell Physiol; 161: 267-276 (1994).

Lawrence WT, Sporn MB, Gorschboth C, Norton JA, Grotendorst GR. The reversal of an Adriamycin(R) induced healing impairement with chemoattractants and growth factors. Ann Surg; 203:142-147 (1986).

LeGrand EK, Burke JF, Costa DE, Kiorpes TC: Dose responsive effects of PDGF-BB. PDGF-AA, EGF and bFGF on granulation tissue in a guinea pig partial thickness skin excision model. Growth Factors; 8:307-314 (1993).

Li CJ, Zhang LJ, Zezube BJ, Crumpacker CS, Pardee AB: Three inhibitors of type I human immunodeficiency virus long terminal repeat directed gene expression and virus replication. Proc Natl Acad Sci USA; 90:1839-1842 (1993).

Li WW, Casey R, Gonzalz EM, Folkman J: Angiostatic steroids potentiated by sulfated cyclodextrins inhibit corneal neovascularization. Invest Opthalmol Vis Sci; 32:2898-2905 (1991).

Linz DN, Garcia VF, Arya G, Ziegler M: Prostaglandin and tumor necrosis factor levels in early wound inflammatory fluid: Effects of parentral omega-3 and omega-6 fatty acid administration. J Pediatric Surg; 29:1065-1070 (1994).

Liotta LA, Rao N, Barsky SH: Tumor invasion and the extracellular matrix. Lab Invest; 49: 636-649 (1983).

Liu JY, Lin SJ, Lin JK: Inhibitory effect of curcumin on protein kinase C activity induced by 12-O-tetradecanoyl-13-acetate in NIH 3T3 cells. Carcinogenesis; 14:857-861 (1993).

Lutomski J, Kedzia B, Debska W: Effect of an alcohol extract and active ingredients from Curcuma longa on bacteri and fungi. Plant Medica; 26:9-19 (1974).

Lysz TW, Arora JK, Lin C, Zelenka P: 12(S)-hydroxyeicosatetraenoicacid regulates DNA synthesis and protooncogene expression induced by epidermal growth factor and insulin in rat lens epithelium. Cell Growth and Diff; 5:1069-1076 (1994).

Maciag T, Cerundalo IS, Ilsley S, Kelly PR, Forand R: An endothelial cell growth factor

from bovine hypothalamus: Identification and partial characterization. Proc Natl Acad Sci USA; 76:5674-5678 (1979).

Mackay AR, Gomez DE, Cottam DW, Rees RC, Nason AM, Thorgeirsson UP: Identification of the 72kDa (MMP-9) gelatinase/Type IV collagenase in preparations of laminin and matrigel. Biotechniques; 15:1048-1051 (1993).

MacNeil S: What role does the extracellular matrix serve in skin grafting and wound healing? Burns; 20:S67-S70 (1994).

Madden JW, Peacock EE: Studies on the biology of collagen during wound healing 1.Rate of collagen synthesis and deposition in cutaneous wounds of the rat. Surgery; 64:288-294 (1968).

Madri JA, Williams SK: Capillary endothelial cell cultures:phenotypic modulation by matrix components. J Cell Biol; 97:153-165 (1983).

Maheshwari RK, Kedar VP, Coon HC, Bharatiya D: Regulation of laminin expression by interferon. J Interferon Res; 11:75-80 (1991a).

Maheshwari RK, Srikantan V, Bharatiya D, Kleinman HK, Grant DS: Differential effects of interferon gamma and alpha on in vitro model of angiogenesis. J Cell Physiol; 146:164-169 (1991b)

Maheshwari RK, Kedar VP, Bharatiya D, Coon HC, Kang HY: Interferon enhances fibronectin expression in various cell types. J Biol Regul Homeost Agents; 4:117-124 (1990)

Martin PM, Wooley JH, McCluskey J: Growth factors and cutaneous wound repair. Prog Growth Factor Res; 4:25-44 (1992).

Matrisian LM: Metalloproteinases and their inhibitors in matrix remodelling. Trends Genetics; 6:121-125 (1990).

Mauch C, Hatamochi A, Scharffetter K, Krieg T: Regulation of collagen synthesis in fibroblasts within a three-dimensional collagen gel. Exp Cell Res; 178:493-530 (1988).

Mazumder A, Raghavan K, Weistein J, Kohn KW, Pommier Y: Inhibition of human immunodeficiency virus type-I integrase by curcumin. Biochem Pharmacol; 49:1165-1170 (1995).

McDonald JA: Fibronectin - a primitive matrix: In the Moecular and Cellular Biology of Wound Repair. (edts) Clark RAF and Henson PM. Plenun Press. New York and London. 405-426 (1988)

McGrath MH: Peptide growth factor and wound healing. Clin Plast Surg; 17:421-432 (1990).

McKenzie RC, Sauder DN: The role of cytokines in inflammation and immunity. J Invest Dermatol; 95:105S-107S (1990).

Merck Index: An encyclopedia of chemicals, drugs, and biologicals. (edts) Budavari S, O'Neil MJ, Smith A, and Heckleman PE. Eleventh edition. Merck and Company, Inc., Rahway, N.J., 2684 (1989).

Mignatti P, Rifkin DB: Biology and biochemistry of proteinases in tumor invasion. Physiological Reviews; 73:161-193 (1993).

Montesano R, Orci L: Tumor promoting phorbol esters induce angiogenesis in vitro. Cell; 42:469-477 (1985).

Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L: Basic fibroblast growth factor induces angiogenesis in vitro. Proc Natl Acad Sci USA; 83:7297-7301 (1986).

Moses MA, Sudhalter J, Langer R: Identification of an inhibitor of neovascularization from cartilage. Science; 248:1408-1410 (1990).

Moses MA, Langer R: A metalloproteinase inhibitor as an inhibitor of neovascularization. J Cell Biochem; 47:230-235 (1991).

Murphy G, Cartwright EC, Sellers A, Reynolds JJ: The detection and characterization of collagenase inhibitors from rabbit tissues in culture. Biochem Biophys Acta; 483:493-498 (1977).

Murphy AN, Unsworth EJ, Stetler-Stevenson WG: Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. J Cell Physiol; 157:351-358 (1993).

Nagase H, Cawston TE, DeSilva M, Barrett AJ: Identification of plasma Kallikrein as an activator of latent collagenase in rhuematoid synovial fluid. Biochem Biophys Acta; 702:133-142 (1982).

Nakadate T, Yamamoto S, Aizu E, Kato R: Inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion and epidermal ornithine decarboxylase activity in mouse skin by palmitoylcarnitine. Cancer Res; 46:1589-1593 (1986).

Nicosia RF, Ottinetti A: Growth of microvessels in serum-free matrix culture of rat aorta: A quantitative assay of angiogenesis invitro. Lab Invest; 63:115-122 (1990).

Norris DA, Clark RAF, Swigart LM, Huff JC, Weston WL, Howell SE: Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes. J Immunol;

129:1612-1618 (1982).

Oikawa T, Hasegawa M, Morita I, Murota SI, Ashino H, Shimamura M, Kiue A, Hamanaka R, Kuwano M, Ishizuka M, Takeuchi T: Effect of 15-deoxy spergualin, a microbial angiogenesis inhibitor, on the biological activities of bovine vascular endothelial cells. Anti-Cancer Drugs; 3:293-299 (1992).

Ono I, Gunji H, Suda K, Iwatsuki K, Kaneko F: Evaluation of cytokines in donor site wound fluids. Scand J Plast Reconstr Hand Surg; 28:269-273 (1994).

Paralkar VM, Vukicevic S, Reddi AH: Transforming growth factor-beta type I binds to Collagen IV of basement membrane matrix: Implications for development. Dev Biol; 143:303-308 (1991).

Pepper MS, Belin D, Montesano R, Orci L , Vassalli JD: Transforming growth factor-beta I modulate basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. J Cell Biol; 111: 743-755 (1990).

Pepper MS, Ferrara N, Orci L, Montesano R: Vascular endothelial growth factor (VEGF) induces the plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem Biophys Res Comm; 181:902-906 (1991).

Pepper MS, Vassalli JD, Wilks JW, Schweigerer L, Orci L, Montesano R: Modulation of bovine microvascular endothelial cells proteolytic properties by inhibitors of angiogenesis. J Cell Biochem; 55:419-434 (1994).

Peschel C, Huber C, Aulitzky WE: Clinical applications of cytokines. La Press Medicale. 23:1083-1091(1994).

Pierce GF, Mustoe TA, Lingelbach J: Platelet derived growth factor and transforming

growth factor beta enhance tissue repair activities by unique mechanisms. J Cell Biol; 109:429-440 (1989).

Postlethwaite AE, Lachman LB, Mainardi CL, Kang AH: Interleukin 1 stimulation of collagenase production by cultured fibroblasts. J Exp Med; 157:801-806 (1983).

Postlethwaite AE, Seyer JM, Kang AH: Chemotactic attraction of human fibroblasts to type I, II and III collagens and collagen-derived peptides. Proc Natl Acad Sci USA; 75:871-875, (1978).

Postlethwaite AE, Kang AH: Collagen and collagen peptide-induced chemotaxis of human blood monocytes. J Exp Med; 143:1299-1307 (1976).

Quaglino D, Nanney LB, Kennedy, Davidson JM: Transforming growth factor-β1 effects in wounds. Lab Invest; 63:306-319 (1990).

Raghow R: The role of extracellular matrix in postinflammatory wound healing and fibrosis. FASEB J; 8:823-831 (1994).

Raychoudhury A, D'Amore PA: Endothelial cell regulation by transforming growth factor beta. J Cellular Biochem; 47:224-229 (1991).

Reddy S, Aggarwal BB: Curcumin is a non-competitive and selective inhibitor of phosphorylase kinase. FEBS Lett; 341:19--22 (1994).

Rijhsinghani K, Greenblatt M, Shubik P: Vascular abnormalities induced by benz(α)pyrene: an in vivo study in the hamster cheek pouch. J Natl Cancer Ins; 41:205-216 (1968).

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI,

Liotta LA, Falanga V, Kehrl JH, Fauci AS: Transforming growth factor type beta: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA; 83:4167-4171(1986).

Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, Yamada SS, Chem WT, Yamada KM,McDonald JA: Transforming growth factor β stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J Biol Chem; 263:4586-4592 (1986b).

Robson MC, Philips LG, Thomason A, Robson LE, Pierce GF: Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. Lancet; 339:23-25 (1992).

Rogelj S, Klagsburn M, Atzmon R, Kurokowa M, Haimovitz A, Fuks Z, Vlodavsky I: Basic fibroblast growth factor is an extracellular component required for supporting the proliferation of vascular endothelial cells and the differentiation of PC 12 cells. J Biol Chem; 109: 823-831 (1989).

Ruoslahti E: Integrins. J Clin Invest; 87:1-5 (1991).

Sakamoto N, Tanaka NG: Mechanism of synergistic effect of heparin and cortisone against angiogenesis and tumor growth. Cancer; 2:9-13 (1988).

Sato N, Nariuchi H, Tsuruoka N, Nishihara T, Beitz JG, Calabresi P and Frackelton R Jr: Actions of TNF and IFN γ on angiogenesis in vitro. J Invest Dermatol; 95:85S-89S (1990).

Sato N, Goto T, Haranaka K, Satomi N, Nariuchi H, Mano-Hirano Y, Sawasaki Y: Actions of tumor necrosis factor on cultured vascular endothelial cells:morphologic modulation, growth inhibition, and cytotoxicity. J Natl Cancer Ins; 76:1113-1121 (1986).

Sato T, Makela M, Kylmaniemi M, Antio-Harmeinen H, Larjava H: Expression of matrix metalloproteinase-2 and -9 during early human wound healing. Lab Invest; 70:176-182, (1994).

Satoskar RR, Shah SJ, Shenoy SG: Evaluation of anti-inflammatory property of curcumin (diferuloyl methane) in patients with postoperative inflammation. Int J Clin Pharmacol Ther Toxicol; 24:651-654 (1986).

Schmitt M, Janicke F, Graeff H: Tumor associated proteases. Fibrinolysis 6 (Supp 4):3-26 (1992).

Schnaper HW, Grant DS, Stetler-Stevenson WG, Fridman R, D'orazi G, Murphy AN, Bird RE, Hoythya M, Fuerst TR, French DL, Quigley JP, Kleinman HK: Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis in vitro. J Cell Physiol; 156:234-246 (1993).

Schonthal A, Herrlich P, Rahmsdorf HJ, Ponta H: Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell; 54:325-334 (1988).

Stephen FO, Hunt TK, Jarvetz E: Effect of cortisone a nd vitamin A on wound infection. Am J Surg; 121:569 (1971).

Setty BN, Dubowy RL, Stuart MJ: Endothelial cell proliferation may be mediated via the production of endogenous lipoxygenase metabolites. Biochem Biophys Res Commun; 144:345-351 (1987).

Shah M, Foreman DM, Ferfuson MWJ: Neutralising antibody to TGF-β1,2 reduces cutaneous scarring in adult rodents. J Cell Sci; 107:1137-1157 (1994).

Shalini VK, Srinivas L: Lipid peroxide induced DNA damage: protection by turmeric (Curcuma longa). Mol Cell Biochem; 77:3-10 (1987).

Shaoshan S: Wound Healing: In International review of connective tissue research. (Edts) Hall DA and Jackson BS. Academy Press, New York; Vol. 9:1-26 (1993).

Sharma OP: Antioxidant activity of curcumin and related compounds. Biochem Pharmacol; 25:1811-1812 (1976).

Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsburn M: Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science; 223:1296-1299 (1984).

Skalli O, Gabbiani G: The biology of the myofibroblast relationshipto wound contraction and fibrocontractive diseases:In Molecular and Cellular Biology of Wound Repair. (Edts) Clark RAF and Henson PM. Plenum Press, New York and London, 373-402 (1988).

Sporn MB, Roberts AB, Shull JH, Smith JM, Ward JM, and Sodek J: Polypeptide transforming growth factor isolated from bovine sources and used for wound healing in vivo. Science; 219:1329-1331(1983).

Srimal RC, Dhawan BN: Pharmacology of diferuloyl methane (Curcumin), a non-steroidal anti-inflammatory agent. J Pharm Pharmacol; 25:447-452 (1973).

Srivastava R and Srimal RC: On the mechanism of action of curcumin, a non-steroidal, anti-inflammatory agent. Ind J Pharmacol; 22:2-6 (1990).

Streuli CH, Bailey N, Bissel MJ: Control of mammalian epithelial differentiation: Basement membrane induces tissue specific gene expression in the absence of cell-cell interaction and its pathophysiological polarity. J Cell Biol; 115:1383-1395 (1991).

Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA Jr, Shepard HM: Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. Science; 230:943-945 (1985).

Sui Z, Salto R, Li J, Craik C, Montellano PR: Inhibition of the HIV-1 and HIV-2 proteases by curcumin and curcumin boron complexes. Bio-organic and Med chem; 1:415-422 (1993).

Tanaka T, Makita H, Ohnishi M, Hirose Y, Wang A, Mori H, Satoh K, Hara A, Ogawa H: Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by dietary curcumin and hesperidin: Comparison with the protective effect of β-carotene. Cancer Res; 54:4653-4659 (1994).

Terranova VP, Rohrbach DH, Martin GR: Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. Cell; 22:719-726 (1980).

Timpl R, Johansson S, Van Delden V, Oberbaumer I, Hook M: Characterization of protease resistant fragments of laminin mediating attachment and spreading of rat hepatocytes. J Biol Chem; 258:8922-8927 (1983).

Toda S, Miyase T, Arichi H, Tanizawa H, Takiyano Y: Natural antioxidants III. Antioxidative components isolated from rhizome of Curcuma longa L. Chem Pharm Bull; 33:1725-1728 (1985).

Tokida Y, Aratani Y, Morita A, Kitagawa Y: Production of two variant laminin forms by endothelial cells and shift of their relative levels by angiostatic steroids. J Biol Chem; 265: 18123-18129 (1990).

Unnikrishnan MK, Rao MNA: Curcumin inhibits nitrite-induced methhemoglobin formation. FEBS Lett; 36:195-196 (1992).

Van De Viel PA, Weitenberg ES, Van Der Pijl, Bloksma N: Factors that define the susceptibility of endothelial cells to tumor necrosis factor and lipid A. Immunopharmacology; 23:49-56 (1992).

Villaschi S, Nicosia RF: Angiogenic role of endogenous basic fibroblast growth factor released by rat aorta after injury. Am J Pathology; 143:181-189 (1993).

Vollberg TM Sr, Margaret DG, Jetten AM: Induction of extracellular matrix gene expression in normal human keratinocytes by transforming growth factor beta is altered by cellular differentiation. J Cell Res; 193:93-100(1991).

Vukicevic S, Luyten FP, Kleinman HK, Reddi AH: Differentiation of canalicular cell processes in bone cells by basement membrane matrix components: Regulation by discrete domains of laminin. Cell; 63:437-445 (1990).

Welch MP, Odland GF, Clark RAF: Temporal relationship of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. J Cell Biol; 110:133-145 (1990).

Woessner JF Jr: Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J; 5:2145-2154 (1991).

Yang EY, Moses HL: Transforming growth factor β_1-induced changes in cell migration, proliferation and angiogenesis in the chicken chorioallantoic membrane. J Cell Biol; 111: 731-741 (1990).