2001

Connective tissue growth factor in human gingiva, and studies of lysyl oxidase processing proteinases

BOSTON UNIVERSITY GOLDMAN

SCHOOL OF DENTAL MEDICINE

DISSERTATION

CONNECTIVE TISSUE GROWTH FACTOR IN HUMAN GINGIVA, AND STUDIES OF LYSYL OXIDASE PROCESSING PROTEINASES

By

MEHMET ILHAN UZEL, DDS

Hacettepe University Faculty of Dentistry

Ankara, Turkey, 1992

Submitted in partial fulfillment of the requirements for the degree

Doctor of Science in Oral Biology

2001

ABSTRACT

The first part of our studies is focused on gingival overgrowth. Gingival overgrowth is characterized by excess extracellular matrix (ECM) accumulation. TGF-β1 regulation of connective tissue growth factor (CTGF) was assessed for the first time in human gingival cells and tissues. CTGF protein is strongly induced by TGF-β1 in human gingival fibroblasts. Exogenous addition of CTGF to gingival fibroblasts stimulates production of lysyl oxidase enzyme activity up to 1.5-fold after 48 hours, and 50 ng/ml CTGF stimulated insoluble collagen accumulation by only 1.5 to 2.0 fold after 4 to18 days of treatment. Thus, although CTGF itself contributes to increased insoluble collagenous extracellular matrix accumulation, CTGF does not mediate more potent effects of TGF-β1. Gingival overgrowth samples obtained from patients undergoing therapy with phenytoin, nifedipine, and cyclosporin A and control tissues from systemically healthy donors were subjected to immunohistochemistry by staining with CTGF, and TGF-β1 antibodies. The results indicate for the first time increased levels of CTGF protein in overgrown gingival tissues, particularly in phenytoin induced gingival overgrowth.

The second part of our studies is focused on the *Bmp1*-related genes. The *Bmp1* gene encodes bone morphogenetic protein 1 (BMP-1) and mammalian Tolloid-like (mTLD), both of which have procollagen C-proteinase activity. Two BMP-1/mTLD-related protease genes named mammalian Tolloid-like 1 and 2 (*Tll1* and *Tll2*) have recently been described. The present study determines and compares the abilities of recombinant BMP-1, mTLD, mTLL-1, and mTLL-2 to process a recombinant pro-lysyl oxidase fusion protein *in vitro*. Results suggest that recombinant BMP-1, mTLD, and

mTLL-1 productively cleave pro-lysyl oxidase at the correct physiological site *in vitro*, and that BMP-1 itself appears to be the most efficient lysyl oxidase processing activity while mTLL-2 was shown to process pro-lysyl oxidase slowly *in vitro*. These studies suggest that pro-lysyl oxidase processing may depend principally on the presence of *Bmp1* gene products, and that mTLL-1, and mTLL-2 may contribute to pro-lysyl oxidase processing.

READER'S APPROVAL

First reader

Philip C. Trackman, PhD

Associate Professor

Department of Periodontology and Oral Biology

Sept. 18, 2001

Date

[redacted]

Signature

Second Reader

Wayne A. Gonnerman, PhD

Associate Professor,

Department of Biochemistry, and Department of Periodontology and Oral Biology

Sept 19, 2001

Date

[redacted]

Signature

CHAIRMAN'S APPROVAL

Chairman

Frank G. Oppenheim, DMD, PhD

Professor,

Department of Periodontology and Oral Biology

September 21, 2001

Date

Signature

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Philip Trackman, who guided me through the exciting field of basic science.

My sincere appreciation to Dr. Frank Oppenheim, and Dr. Thomas E. Van Dyke, and all of the professors of this department who, in different ways, provided me with advice and encouragement.

I am in debt with Dr. Dana Graves, and Dr. Salomon Amar for providing us their equipments for the sectioning and analysis of our gingival tissue samples, with FibroGen (California) for generously providing us with rCTGF and its antibody, with Dr. Alpdogan Kantarci for his cooperation and efforts for the completion of our studies, and with Dr. Daniel Greenspan (University of Wisconsin School of Medicine) for providing BMP-1 and related enzymes.

I would specially thank to Dr. Ilter Uzel, the Dean of Cukurova University Faculty of Dentistry for unconditionally supporting my studies, and Cukurova University for mostly funding my studies in the United States.

I am grateful to my parents for their inestimable support and love.

DEDICATION

To Guzin my wife,

And to Doruk my son,

With love

TABLE OF CONTENTS

LIST OF FIGURES

TABLE

LIST OF ABBREVIATIONS

BAPN: β-aminopropionitrile

bFGF: basic fibroblast growth factor

BMP-1: bone morphogenetic protein 1

Bmp1: bone morphogenetic protein 1 gene

BSA: bovine serum albumin fraction V

CEJ: cemeto-enamel junction

COL1A1: α 1type 1 collagen

CTGF: connective tissue growth factor

DMEM: Dulbecco's Modified Eagle's Medium

DPP: Decapentaplegic

ECM: extracellular matrix

EDTA: ethylene diamietetraacetic acid

EGF: epidermal growth factor

bFGF or FGF2: basic fibroblast growth factor

IL-1: α: interleukin one alfa

IL-6: interleukin six

mTLD: mammalian Tolloid

mTLL-1: mammalian Tolloid-like 1

mtll-1: mammalian Tolloid-like 1 gene

mTLL-2: mammalian Tolloid-like 2

mtll-2: mammalian Tolloid-like 2 gene

MMP-1: matrix metalloproteinase one

Na_2Ppi: disodium pyrophosphate

NBS: newborn serum

PBS: phosphate buffered saline

PCP: procollagen c terminus protease

PCPE: Procollagen C-proteinase enhancer

PDGF: platelet derived growth factor

PHE: phenytoin-induced gingival overgrowth

PMSF: phenymethylsulfonyl fluoride

RT-PCR: reverse transcriptase polymerase chain reaction

SDS: sodium dodeclyl sulfate

SOG: Short Gastrulation

ssDNA: salmon sperm DNA

TGF-β1: transforming growth factor beta one

TNF-α : tumor necrosis factor alfa

INTRODUCTION

Gingival overgrowth is characterized by an accumulation of excess gingival tissue due to an increase in extracellular matrix and cellular elements without excessive periodontal bone loss. This can result from a variety of inflammatory and/or fibrotic conditions that may be influenced by hormonal, nutritional, and other systemic factors (Carranza 1996). The hereditary condition known as familial fibromatosis characterized by gingival overgrowth has also been identified (Hassell and Hefti, 1991; Tipton *et al.*, 1997). The most common forms of gingival overgrowth occur as side effects of therapy with certain antihypertensive calcium channel blockers, the immunosuppressive drug cyclosporin A, and the anti-seizure drug phenytoin (Hassell and Hefti, 1991). Recently, increased levels of several cytokines have been found in drug-induced gingival overgrowth tissues. Thus, bFGF, TGF-β, IL-6, PDGF, and IL-1α are all elevated in human gingival overgrowth tissues in vivo (Dill *et al.*, 1993; Iacopino *et al.*, 1997; Plemons *et al.*, 1996; Saito *et al.*, 1996; Williamson *et al.*, 1994). The activity, mechanisms and mediating factors that stimulate production of different components of the extracellular matrix initiated by these cytokines in gingival fibroblasts is only partially understood, but seem likely to play important roles in all forms of gingival overgrowth. Interestingly, evidence for a contributing role of increased autocrine TGF-β mediated stimulation of extracellular matrix production by human gingival fibroblasts in familial gingival fibromatosis has been reported (Tipton and Dabbous, 1998).

TGF-β1 is a fibrogenic factor that stimulates extracellular matrix accumulation in different tissues. For example, TGF-β1 is known to stimulate collagen production and mature insoluble collagen accumulation by fibroblasts, osteoblasts and keratinocytes (Centrella *et al.*, 1992; Meisler *et al.*, 1997). Extracellular enzymes that post-translationally modify procollagen in

the formation of a mature insoluble collagenous extracellular matrix have been shown to be up-regulated in fibroblasts and osteoblasts by TGF-β1. For example, procollagen C-proteinase (Lee *et al.*, 1997), and the extracellular enzyme lysyl oxidase (Boak *et al.*, 1994; Feres-Filho *et al.*, 1995; Shanley *et al.*, 1997) are up-regulated by TGF-β1 in a variety of fibrogenic cell types. Lysyl oxidase catalyzes the final known enzymatic step required for cross-linking and insolubilization of collagen and elastin precursors in the formation of a mature and functional extracellular matrix (Kagan, 1986; Kagan and Trackman, 1991). It has been suggested that lysyl oxidase may be a key regulatory enzyme in controlling extracellular matrix accumulation, as a cross-linked matrix is less susceptible to proteolysis (Kagan, 1986).

Among the genes stimulated by TGF-β1 is connective tissue growth factor (CTGF) (Igarashi *et al.*, 1993; Oemar and Luscher, 1997). CTGF is strongly induced by TGF-β in skin and kidney fibrogenic cells, and has been found at elevated levels in several different hyperplastic or fibrotic tissues including skin, and aorta (Frazier *et al.*, 1996; Igarashi *et al.*, 1996; Igarashi *et al.*, 1995; Oemar *et al.*, 1997). CTGF production is also increased by dexamethasone in murine fibroblasts, and decreased by TNF-α (Dammeier *et al.*, 1998). Interestingly, CTGF production is not stimulated by TGF-β1 in large vessel endothelial cells indicating that CTGF is not universally stimulated by TGF-β's in all cell types. Some of the biological effects of TGF-β, including stimulation of extracellular matrix accumulation, may be mediated by CTGF (Frazier *et al.*, 1996). The CTGF protein is a 42 kDa cysteine-rich glycoprotein and has conserved structural motifs including a von Willebrand factor-like repeat domain, and a thrombospondin-like repeat domain. These features suggest that in addition to stimulating cells by receptor mediated events (Nishida *et al.*, 1998), CTGF may also interact with other structural components of the extracellular matrix, and exert biological effects (Oemar

and Luscher, 1997). In addition to its effects on extracellular matrix production, CTGF is mitogenic, and has recently shown to promote angiogenesis in vivo (Babic *et al.*, 1999; Nakanishi *et al.*, 1999). The hypothesis of the present work is that CTGF may be expressed at elevated levels in hyperplastic gingiva and may contribute to development of gingival overgrowth and excess extracellular matrix accumulation.

Lysyl oxidase catalyzes the oxidative deamination of peptidyl-lysine in elastin precursors, and lysine and hydroxylysine residues in collagen, to form peptidyl-α-aminoadipic-δ-semialdehyde and peptidyl-δ-hydroxy-α-aminoadipic-δ-semialdehyde residues, respectively. These aldehydes then undergo non-enzymatic reactions resulting in the cross-linkages known to be critical in the formation of mature insoluble elastin and collagens (Kagan, 1986; Kagan and Trackman 1991). Lysyl oxidase is a copper-dependent enzyme, and is specifically inhibited by β-aminopropionitrile (BAPN). Connective tissue abnormalities known as lathyrism resulting from *in vivo* inhibition of lysyl oxidase activity by either BAPN-feeding or feeding animals a copper deficient diet have been described (Rucker *et al.,* 1975). Abnormalities include malformed and weak bones, as well as increased development of hernias (Bedell-Hogan *et al.,* 1997). Lysyl oxidase is synthesized as a 50 kDa glycoprotein and is processed extracellularly to produce the 30 kDa molecular form known to be active (Trackman *et al.*, 1992). The sequence of the proteolytic processing site in pro-lysyl oxidase resembles that of the fibrillar procollagen C-terminal pro-peptide processing sites cleaved by procollagen C-proteinase. Moreover, preparations of highly purified procollagen C-proteinase activity have been shown to process pro-lysyl oxidase at the correct physiological site, and procollagen C-proteinase has been implicated as the principal lysyl oxidase processing activity in fibrogenic cell cultures (Panchenko *et al.,* 1996). In mammals, procollagen C-proteinase activity is provided by products

of the *Bmp1* gene (Kessler *et al.,* 1996; Scott *et al.,* 1999), which encodes alternatively spliced mRNA's for bone morphogenetic protein 1 (BMP-1) and mammalian Tolloid (mTLD) (Takahara *et al.,* 1994). The mTLD protein product contains a longer C-terminus than BMP-1, resulting in a domain structure identical to that of the *Drosophila* protein Tolloid. Domains in BMP-1, mTLD, and *Drosophila* Tolloid include the astacin-like protease domain, and EGF-like domains and CUB domains that may mediate binding to other extracellular proteins (Prockop *et al.,* 1998; Hulmes *et al.,* 1997). *Drosophila* Tolloid plays an important role in pattern formation during embryogenesis by cleaving the secreted protein Short Gastrulation (SOG), which forms latent complexes with the transforming growth factor β-related protein Decapentaplegic (DPP) (Marques *et al.,* 1999). BMP-1 may play a similar role in mammalian embryogenetic patterning, since it cleaves the SOG vertebrate homologue chordin, which binds and inactivates the DPP vertebrate homologues BMP-2/BMP-4 (Scott *et al.,* 1999; Mullins, 1998). Although mammalian BMP-1 and mTLD both are procollagen C-proteinases, only BMP-1 hydrolyzes chordin, indicating that *Bmp1* gene products have different substrate specificities and some different biological functions (Scott *et al.,* 1999).

The mammalian *Bmp1* gene is a member of a multigene family and two genetically distinct mammalian Tolloid-related proteases, mammalian Tolloid-like 1 and 2 (mTLL-1 and mTLL-2), have recently been cloned and expressed (Scott *et al.,* 1999). Interestingly, despite highly similar sequences and domain structures, the substrate specificities of BMP-1, mTLD, mTLL-1, and mTLL-2 differ. BMP-1, mTLD, and mTLL-1 all have readily detectable procollagen C-proteinase activity, whereas mTLL-2 does not. BMP-1 and mTLL-1 readily cleave chordin, and mTLD and mTLL-2 do not (Scott *et al.,*1999). It has yet to be determined whether differences in specificity occur with other substrates, such as lysyl oxidase.

Mice homozygous null for the *Bmp1* gene have impaired ossification of calvaria, herniation of the gut, do not survive beyond birth, but have grossly normal axial and appendicular skeletons (Suziki *et al.*,1996). *In vitro*, fibroblasts from *Bmp1* null embryos accumulate less insoluble collagen, and collagen fibrils are not normal in appearance. Procollagen processing is diminished and results in accumulation of intermediates that retain the C-propeptide, in addition to low amounts of fully processed tropocollagen (Suziki *et al.*,1996). These results indicate that in *Bmp1* null embryos and fibroblasts related proteases are able to partially, but not fully, compensate for the loss of the procollagen C-proteinase activity normally provided by BMP-1 and mTLD. Interestingly, the *Bmp1* null phenotype that includes gut herniation and skeletal abnormalities, particularly calvaria maturation appears similar to lathyrism (Selye,1957). As noted above, lathyrism is caused by a deficiency in lysyl oxidase enzyme activity (Kagan,1986; Kagan and Trackman,1991) .

The lysyl oxidase pro-enzyme processing activities of the different BMP-1-related proteinases have not previously been measured, nor has the major enzyme responsible for processing of pro-lysyl oxidase *in vivo* been identified. The present study compares the ability of BMP-1, mTLD, mTLL-1, and mTLL-2 enzymes to process pro-lysyl oxidase *in vitro*. Results indicate that mTLL-1, and mTLL-2 may contribute to lysyl oxidase processing, but that lysyl oxidase processing may depend principally on BMP-1. We show that pro-lysyl oxidase is the first known substrate for mTLL-2.

LITERATURE REVIEW

Drug Induced Gingival Overgrowth

Phenytoin Induced Gingival Overgrowth

Drug-induced gingival overgrowth was first reported in 1939 and resulted from chronic administration of the anti-epileptic agent, phenytoin. According to this article the incidence of gingival overgrowth was 57% among patients undergoing therapy with phenytoin (Kimball 1939). Ever since there have been many studies published about the incidence of drug induced gingival overgrowth, and phenytoin induced gingival overgrowth in particular has been extensively documented. Phenytoin-induced gingival overgrowth was evaluated in a cross-sectional, epidemiological study among 60 epileptic patients. Gingival lesion severity was statistically compared with other clinical, laboratory, and histopathological findings. Obvious gingival overgrowth was observed in 50% of 60 patients. Even though positive correlations were detected between overgrowth severity and oral debris, calculus accumulation, plaque score, gingival inflammation, and probing depth, no strong correlation was found between lesion severity and patient age, mouth breathing, daily drug dose, plasma phenytoin level, or duration of drug intake (Penarrocha-Diago *et al.,*1990). The incidence of phenytoin-induced gingival overgrowth among epileptic patients and healthy patients was later evaluated investigating relationships with clinical parameters including plaque score, gingival index, and the amount of overgrown gingiva. The correlation between plaque score and gingival overgrowth was high in the patients taking phenytoin. The incidence of clinically very significant gingival overgrowth was around 13% (Thomason *et al.,* 1992). In another study, 134 patients taking phenytoin were evaluated in a community based cross sectional study. The investigators used a grading system

indicating the severity of gingival overgrowth (Angelopulos *et al.,* 1972). The overall frequency of phenytoin induced gingival overgrowth was around 40%. Among these patients, 68% showed mild, and 32% showed moderate or severe gingival overgrowth. The mean plaque index was higher but not statistically significant in the patients exhibiting drug-induced gingival overgrowth even though the moderate to severe gingival overgrowth group had higher plaque index scores than the mild drug-induced gingival overgrowth (Casetta *et al.,*1997). Interestingly, in an earlier study, a plaque control program was applied for two years on 20 children taking phenytoin, and no reduction of drug induced gingival overgrowth was accomplished (Dahlof and Modeer, 1986). Taken together, studies suggest that 40 to 50% of the patients taking phenytoin show drug-induced gingival overgrowth to varying degrees. It is more likely that the plaque accumulation has a minor role in this mechanism, and that phenytoin may trigger a biological cascade causing gingival overgrowth. Since in general one of the main constituents of a fibrotic extracellular matrix is collagen, it is important to understand whether there is evidence in the literature linking increased collagen synthesis and accumulation with drug induced gingival overgrowth. Experiments performed with fibroblasts generated from the overgrown tissues from epileptic patients taking phenytoin had twice the level of protein synthetic activity compared to fibroblasts taken from non-epileptic control patients as well as from age-matched epileptic patients taking phenytoin who did not have drug-induced gingival overgrowth. Cells from the overgrown tissue synthesized collagen as 20% of total protein content. This amount was only 11% for cells taken from the control patients (Hassell *et al.,* 1976). The effects of exogenously added phenytoin on human gingival fibroblast collagen synthesis were also studied. Seventeen healthy individuals, volunteering for the study, were surgically excised tissue specimens from the interdental papilla between the premolars. The samples were immediately minced and

subjected to tissue culturing in order to generate fibroblasts. Then the generated cells were cultured for 24 hours with control or medium containing 5 μg/ml of phenytoin. After 24 hours, the cells were subjected to pulse labeling with ^{3}H-proline for 25 hours in order to evaluate protein and collagen synthetic activity and accumulation. Collagen synthesis was estimated by the use of chromatographically purified bacterial collagenase. Seven out of seventeen cultures increased collagen synthesis in response to phenytoin. It is not easy to make conclusions based on the results given, because even though certain cell cultures showed statistically significant increased collagen synthesis, some others showed statistically diminished collagen synthesis. The suggestion of the authors about the cell line specificity may be reasonable, but still, there were no data presented representing the mean values. Moreover, most of the data have been generated after one set of experiments, and have not been reproduced by other group of investigators (Hassell and Gilbert, 1983). It is therefore unclear whether phenytoin directly stimulates gingival fibroblasts to produce more collagen, or whether indirect mechanisms are more important.

The microscopic evaluation of the biopsy specimens from patients taking phenytoin and comparison to the control specimens has great importance. Samples from 12 patients taking phenytoin with moderate to severe gingival overgrowth were fixed and sectioned and compared with the samples from five age-matched healthy individuals and with samples taken from five age matched control patients. Direct counting of fibroblast nuclei and stereological point-counting procedures were performed on the samples sectioned in 5μm thickness. The results show no difference in between the groups in terms of hypercellular activity, as it had been previously suggested and summarized above, or collagen per unit tissue (Hassell *et al.,* 1976). Even though the results show that collagen amounts may not be different between the phenytoin

group and control groups by microscopic evaluation, no technique such as immunohistochemistry to specifically identify different types of collagen or other ECM molecules was utilized. Moreover the finding of similar nuclei counts per unit area may allow the conclusion that phenytoin induced gingival overgrowth is not hyperplasia, since gingival hyperplasia is a condition describing the gingival overgrowth by the increased number of cells rather than the increased amount of ECM components. These studies invalidated the term "hyperplasia" to describe a phenytoin-induced gingival overgrowth (Hassell *et al.,* 1978). In the same context, the amounts of extracellular matrix components such as collagen, type III and type IV, vessels, fibroblasts, fibronectin, and elastic fibers were investigated in drug induced gingival overgrowth caused by the usage of cyclosporin, phenytoin and nifedipine and compared with the control group. The study employed histological and immunohistochemical analysis. In general, histologic investigation showed similarities in gingival overgrowth induced by different drugs. When the same samples were evaluated by using immmunohistochemistry methods, the fibroblasts were significantly different in phenytoin group but not in the nifedipine and cyclosporin groups than it was in healthy gingiva and chronic gingivitis, but the areas of collagen and fibronectin were significantly greater in the nifedipine group than in the other groups (Bonnaure-Mallet *et al.,* 1995).

Other efforts to describe the extracellular matrix (ECM) components in drug induced gingival overgrowth have been made. Proteoglycans, and glycosaminoglycans were evaluated in the papillae of the children taking phenytoin and control tissues obtained from age-matched healthy children. Biochemical extraction of the proteoglycans and glycosaminoglycans was done and evaluated by gel electrophoresis and HPLC. The results showed that normal gingiva contained 0.8% uronic acid per dry tissue weight where as phenytoin induced gingival

overgrowth samples contained 2.1%. Thus, this study supports that the composition of the ECM is changed in phenytoin induced gingival overgrowth (Dahlof *et al.*, 1986). The fibronectin distribution in the healthy, inflamed and hyperplastic human gingiva was studied. In the healthy gingiva, fibronectin had a fibrillar structure in the lamina propria. In the phenytoin induced gingival overgrowth samples fibronectin showed parallel fibers in the inflamed specimens and had thin fibers with variable length penetrating in the basement membrane. Higher length and the parallel distribution of the fibronectin in the cyclosporin A induced gingival overgrowth samples showed that there was a difference from the phenytoin induced DGO specimens. Moreover, fibronectin showed a cloud-like microfibrillar network in the nifedipine induced gingival overgrowth. All these observations have great importance in terms of distinguishing drugs that cause similar clinical appearance, but possibly different kind of biochemical effects (Romanos *et al.*, 1992).

The Evaluation of Phenytoin Induced Gingival Overgrowth with Animal Studies

In addition to the studies that have focused on *in vitro* experiments, and the epidemilogy of gingival overgrowth, a few studies have investigated biological mechanisms. One of these studies was done using ferrets administrated phenytoin (Hall *et al., 1991*). The experimental protocol consisted of 32 male ferrets being de-scented and castrated and fed a soft diet four weeks before the experiments. Then when the ferrets were 14 weeks old the experimental procedure began. The study consisted of control, banded control, phenytoin, and banded phenytoin groups. The ferrets in the banded groups, received wire mesh bands attached to the maxillary teeth to cause plaque accumulation and to induce gingival inflammation. Phenytoin

groups had 40 mg/kg phenytoin mixed into the animals daily food. This dosage results in a plasma concentration of phenytoin 16 μg/ml, which is equivalent to the human clinical therapeutic dose (10-20 μg/ml). Biopsies were taken at one and five month time points. Histological quantitation was done by evaluating the area adjacent cemeto-enamel junction (CEJ), the most superficial portion of the gingival crest, and the area around free gingival groove. The results showed healthy gingiva in the control animals despite increased inflammation in the banded group animals. Histological appearance was consistent with healthy primates in control animals. There was some crestal epithelial proliferation in the banded control animals. The gingiva in the phenytoin-fed animals showed bucco-lingual enlargement compared to the control animals. An inflammatory response was seen in the banded phenytoin group. Microscopic investigation revealed that collagen was more dispersed and irregular in the phenytoin-fed animals. The amount of collagen was increased at the free gingival groove-crest area in phenytoin administrated animals. Banding did not have any effects on the severity of the phenytoin induced overgrowth. Even though this study showed the strength of phenytoin in inducing the gingival overgrowth, it also contradicts some findings claiming increased plaque contributing to the etiology of drug induced gingival overgrowth. According to the same study, the relative volume of interstitial material (ground substance) was significantly increased after phenytoin administration, despite no changes in cell numbers. The authors claimed that the ultrastructure of fibroblasts was changed. These cells showed a decrease in the relative volume of phagosomes, even though the rough endoplasmic reticulum and Golgi zones, which are the organelles related to biosynthesis in the cells, were not changed. (Hall *et al.,* 1982). Phenytoin induced gingival overgrowth was also investigated by using a mongrel cat model in which time-dependent changes in developing phenytoin-induced lesions were investigated by measuring the

number of fibroblasts per unit of tissue in papilla biopsies collected over a 3-month period. Eighteen cats between the ages of 9-24 months were used in these experiments. The experimental group received 4 mg/kg of phenytoin daily. The biopsies were collected at intervals over 3 months. Four out of eight cats administered phenytoin showed clinical gingival overgrowth. The number of fibroblasts per unit of tissue increased dramatically at 6 and 8 weeks. However, later on, when the lesions matured, the fibroblast-to-matrix ratio returned to initial levels after three months. Despite the interesting findings, the dosage of phenytoin administration was relatively low, and the species used in two studies were different. It is therefore unclear whether phenytoin induced gingival lesions in this study represent hyperplasia or overgrowth (Hassell et *al.,* 1982).

An ultrastructural study of rat hyperplastic connective tissue revealed increased myofibroblasts compared to the control samples. In this study, eight rats were injected daily with 100 mg / kg of phenytoin, and eight rats were used as controls. No myofibroblasts were detected in the samples taken from control animals, whereas numerous myofibroblasts were identified in the phenytoin treated animals. In this article, the study was supported by several electron micrographs but no quantitative analyses of the samples were presented. (Dill and Iacopino, 1997). Myofibroblasts are known to produce high amounts of extracellular matrix, and contribute to fibrosis (Gabbiani, 1994).

Cytokine Involvement In Drug Induced Gingival Overgrowth

Gingival overgrowth is caused by phenytoin, and also by cyclosporin A and nifedipine. Since different drugs may trigger different kinds of biochemical effects to various degrees, it seems possible that different levels of cytokines also play important roles in drug induced gingival overgrowth. In that context immunohistochemical analyses of TGF-β1, TGF-β2 and TGF-β3, and bFGF, their receptors and heparan sulphate glycosaminoglycan was studied in nifedipine- induced and phenytoin-induced gingival overgrown tissues, as well as in control tissue samples. Positive staining with antibodies against TGF-β1, bFGF, the receptors of these two growth factors and glycosaminoglycan were localized in the lamina propria of overgrown gingival tissues. All of the samples were evaluated per unit area. In phenytoin induced gingival overgrowth samples, TGF–β1 was present in 8.4+3.7 cells, bFGF was present in 19.5+8.7 cells, TGF-β1 receptor was present in 28.9+6.9, and the bFGF receptor was present in 23.3+6.5 cells. In nifedipine induced gingival overgrowth samples, TGF-β1 was present in 7.2+3.3 cells, bFGF was present 14.5+10.4 cells, TGF-β1 receptor was present in 25.7+8.8 cells, and the bFGF receptor was present in 23.3+6.5 cells. Less positive staining was observed in the control specimens, TGF-β1 was present in 0.5+0.8 cells, bFGF was present in 4.2+1.9 cells , TGF-β1 receptor was present in 7.9+3.2 cells, and bFGF receptor was present in 6.4+3.5 cells. Despite the high error margin in some of the data, the increased amounts of TGF-β, bFGF, their receptors, and glycosaminoglycan may be related to the pathogenesis of drug-induced gingival overgrowth, as well as the other components of ECM such as collagen (Saito *et al.,* 1996). In addition to the positive staining for TGF-β1 in drug-induced gingival overgrowth samples, it was also detected at the tips of the dermal papillae of the overgrown gingiva. It was shown *in vitro* that the fibroblasts derived from overgrown gingival tissue are more responsive to TGF-β1

than normal gingival fibroblasts when cultured in type I collagen gel (James *et al.,* 1998).This finding may be important with regard to a possible growth factor stimulation of gingival fibroblasts harvested from overgrown gingivae, eventually causing gingival overgrowth.

Production of PDGF-B and IL-1β in tissues samples of the phenytoin treated patients showing gingival overgrowth was compared with control patients with or without gingival inflammation. Quantitative competitive reverse transcription polymerase chain reaction (QC-RTPCR) techniques were utilized to measure PDGF-B and IL-1 beta mRNA levels in experimental groups. Cyclosporin A caused a dramatic increase in PDGF levels but did not cause any changes in IL-1β levels. Patients treated with phenytoin with gingival overgrowth had a significant increase in PDGF-B mRNA compared to controls. Patients with severe gingival inflammation also showed a significant increase in PDGF-B mRNA. However, phenytoin induced PDGF-B about six times more than in patients having severe gingival inflammation without phenytoin therapy. The clinical samples were also investigated for macrophage phenotype by using immunohistochemistry methods. There was a significant increase in the reparative/proliferative macrophage phenotype in the hyperplastic gingival tissue. It is then reasonable to claim that one cytokine is likely to be more expressed in the gingival overgrowth caused by phenytoin (Iacopino *et al.,* 1997). Considering that PDGF-B may play an important role in the drug-induced gingival overgrowth, its gene expression was evaluated in tissue samples from patients taking cyclosporin A. In situ hybridization and immunocytochemical analysis showed significant upregulation of PDGF-B gene expression in tissues from the patients taking cyclosporin A. Macrophages were shown to produce PDGF-B in these samples. It was suggested that cyclosporin A induced gingival overgrowth was caused by enhanced macrophage PDGF-B gene expression rather than an increase in the number of PDGF-B producing

macrophages (Plemons *et al.,* 1996). In another human study, IL-1β and IL-6 levels of the peripheral blood mononuclear cells in normal, inflamed, and cyclosporin A induced gingival overgrowth tissue samples were investigated. It was suggested that cyclosporin A regulated expression of cytokine such as IL1β and IL-6 in gingival tissue (Myrillas *et al.,* 1999).

The androgen or its biologically active metabolite 5 alpha- dihydrotestosterone (DHT) may stimulate ECM synthesis in connective tissue and bone. The formation of DHT from testosterone in response to phenytoin, interleukin-1, and epidermal growth factor (EGF) was investigated. EGF and IL-1α present in inflammatory exudate may have effects on phenytoin-induced gingival overgrowth suggesting that steroid metabolism may be another mechanism triggering gingival overgrowth (Soory *et al.,* 1997).

Another attempt to understand drug induced gingival overgrowth was the evaluation of prostaglandin E_2 formation in human gingival fibroblasts generated from patients taking phenytoin. Gingival tissue samples were taken before and nine months after the administration of phenytoin. In the fibroblastic cultures from the samples taken before the drug administration, IL-1α, IL-1β, and TNFα stimulated formation of PGE_2 in a dose-dependent manner. In the fibroblasts from the samples taken nine months after the drug administration, IL-1α, IL-1β, and TNFα stimulated the significantly higher formation of PGE_2 comparing to the pretreatment phase. The authors suggested that the administration of phenytoin results in an upregulation of prostanoid formation in gingival fibroblasts related to the phospholipase A_2 activity and to an increased cyclooxygenase activity (Modeer *et al.,* 1992).

Other Drugs Inducing Gingival Overgrowth

As it is mentioned in the sections above, drugs other than phenytoin are known to cause gingival overgrowth. As it is stated in the materials and methods, we also have investigated cytokine levels in gingival overgrowth tissue samples taken from the patients taking drugs other then phenytoin. It is important to determine whether different drugs cause drug-induced gingival overgrowth through the same mechanism as phenytoin, and whether specific cytokines may play an unique role in the gingival overgrowth caused by a particular drug.

Cyclosporin A is an immunosuppressant, and causes gingival overgrowth. It has been suggested that individual hypersensitivity of each patient to cyclosporin A may contribute to the incidence of gingival hyperplasia. This study is based on the electron microscopic findings of gingival biopsies taken from only four transplant patients (Deliliers *et al.,* 1986).

Cyclosporin A-induced gingival overgrowth pathogenesis was evaluated *in vitro* in human gingival fibroblasts in order to investigate effects on ^{3}H thymidine incorporation, and on collagen production, and collagen mRNA levels in fibroblast cultures obtained from normal human gingiva. The concentrations of 100, 500, and 1000 ng/ml cyclosporin A did not modify thymidine incorporation after 24 and 72 h of incubation. However, after 24 hours the level of ^{3}H proline-containing proteins significantly increased in the medium, and cyclosporin A increased the production of α–procollagen chains by three fold. It was suggested that cyclosporine A stimulated collagen production without changes in the DNA synthesis (Schincaglia *et al.,* 1992).

The correlation between the severity of gingival overgrowth and blood cyclosporin A levels as well as its dose dependent effects were investigated in rats given oral cyclosporin A. The blood levels of cyclosporin A increased with dose, and overgrowth was more severe in buccal than in lingual gingiva. A positive correlation between gingival sulcus depth and the blood cyclosporin A level was found. The overgrown gingiva consisted of a thickened epithelial layer and an accumulation of subepithelial fibrous connective tissue components without important changes in their proportion (Morisaki *et al.*,1997). Another in vivo study investigated the effects of cyclosporin A on rat gingiva. The overgrowth of the gingival connective tissue resulted from vasodilatation, and increased volume of the extracellular matrix, and from increased epithelial thickness. All of these morphological changes may be due to cell hypertrophy in the keratinized epithelium and cell hyperplasia in the junctional epithelium (Ayanoglou *et al.,* 1999). The distribution of the peripheral blood leukocytes in a group of renal transplant patients undergoing cyclosporin A therapy was analyzed, and possible correlations were analyzed between periodontal and pharmacological variables and lymphocyte subpopulations, natural killer cells, and monocytes. Several previously unsuspected cells and accessory activation mechanisms for T lymphocytes were suggested to play a role in the pathogenesis of drug induced gingival overgrowth (Cebeci *et al.*, 1998). In addition to phenytoin, and cyclosporine A, it was shown that nifedipine can also cause gingival overgrowth showing clinical and histological similarity to phenytoin (van der Wall *et al.,* 1985). Drug induced gingival overgrowth was also investigated in patients previously taking nifedipine and then switching to a dihydropyridine derivative (isradipine) with a low incidence of gingival overgrowth. Probing depth, gingival margin, gingival thickness, plaque index, and gingival index were evaluated. Patients were randomized for either continued treatment with nifedipine, or for

treatment with an equivalent dose of isradipine. It was found that in hypertensive patients with nifedipine induced gingival overgrowth, switching hypertensive therapy to isradipine may result in a regression of the problem in conjunction with meticulous oral hygiene (Westbrook *et al.*,1997).

Twenty-four dentate patients who received verapamil, which is a calcium channel blocker, for more than one year were identified after reviewing the records of 5,000 dental patients for history of verapamil use between 1987 and 1990. Among these 24 patients gingival overgrowth occurred in one patient. The overgrowth was associated with drug dosage, bacterial accumulation, and gingival inflammation. Histologically the gingival overgrowth was similar to that caused by phenytoin, cyclosporin, and other calcium channel blockers. The authors suggest that gingival overgrowth caused by verapamil occurred less frequently than nifedipine-induced gingival hyperplasia (Miller and Damm, 1992)

Amlodipine, which is another calcium channel blocker, has also been reported to cause gingival overgrowth after its chronic use. Gingival changes occurred within 3 months of administration of the drug and apparently were related to a patient's periodontal condition. A trace of amlodipine was also detected in the crevicular fluid (Seymour *et al.*, 1994). Recent studies indicate a low frequency of drug-induced gingival overgrowth in patients taking amlodipine (Ellis *et al.*, 1999).

In dogs, presence of plaque and gingival inflammation was shown to be an important cofactor in gingival overgrowth caused by the administration of nitrendipine, an antihypertensive dihydropyridine. Histopathology studies showed that the main difference in morphology was that areas of non-infiltrated connective tissue in test animals showed an increase in vascularity and seemed to be less dense compared to the dense tissue in control specimens. Essentially, the 20-

week period of drug administration causes significant overgrowth of the gingival tissues even tough the composition of the gingival tissue is apparently normal (Heijl *et al.*,1989).

Connective Tissue Growth Factor

Discovery, Structure, and Different Forms of Connective Tissue Growth Factor

CTGF was first discovered during analysis of proteins secreted by human vascular endothelial cells, using anti-PDGF IgG and antibodies specific for the A or B chain peptides of PDGF. The main PDGF immunorelated molecule was a monomer of approximately of 36-38 kDa. The newly discovered protein was then cloned using the anti-PDGF antibody to probe an expression library. This resulted in the isolation of cDNA's with an open reading frame encoding a 38-kDa cysteine-rich secreted protein named connective tissue growth factor (Bradham *et al.*, 1991). The CTGF gene as well as its family members nov, cyr61, wisp1 and wisp2 encode secreted proteins with conserved domains including an IGF binding domain, a von Willebrand factor repeat, and a thrombospondin repeat as well as insulin like growth factor binding protein motifs in their NH_2 termini (Oemar and Luscher1997; Kim *et al.*, 1997). Moreover, highly truncated, heparin binding forms of 10, 16, and 20 kDa CTGF proteins were found in uterine luminal fluids (Brigstock *et al.*, 1997). In vitro experiments revealed cell-associated and soluble forms of CTGF as well as a 10 kDa active form, all produced by fibroblasts. Human foreskin fibroblasts as well as mouse 2555 and 2472 fibroblasts and balb/c mouse 3T3 cells were cultured in order to identify the CTGF fragments. Cells were incubated in serum free medium when they had 70 to 80% confluency. The media were subjected to high performance liquid chromatography (HPLC) in order to purify the CTGF fragments. CTGF fragments (10-12 kDa)

were identified in all of the cell lines investigated. Furthermore, these fragments were mitogenic for cells. Mouse cells were also pulse labeled with ^{35}Cys/Met and the presence of both 38 kDa CTGF and lower molecular weight fragments was verified by immunoprecipitation using human CTGF antibody. (Steffen *et al.,* 1998). Moreover, pig uterine luminal flushings were analyzed and the processing of 38kDa CTGF was found to be concentration and time-dependent. Levels of 10 to 20 kDa forms of CTGF peaked on day 12 to16 of pregnancy and were highly correlated with the levels of proteolytic activity for 38 kDa CTGF (Ball *et al.,* 1998).

The Mediation of TGF-β Effects on Extracellular Matrix by CTGF

The finding that human foreskin fibroblasts produce high levels of CTGF mRNA and protein after stimulation by transforming growth factor beta (TGF-β) suggests that CTGF could possibly mediate effects of TGF-β. Moreover, cytokines such as PDGF, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF or FGF2) did not stimulate CTGF production. The stimulation of CTGF gene expression by TGF-β could not be blocked by cycloheximide. This showed that the synthesis of other proteins was not required, and indicated that TGF-β directly stimulates CTGF production. In order to confirm and evaluate this cascade human newborn foreskin fibroblasts were cultured and treated with TGF-β, PDGF, EGF, or FGF. Cells were harvested , lysed, and subjected to Western blotting using anti-PDGF IgG. Only TGF-β treated cells expressed the 38 kDa PDGF-related peptide. As it was mentioned above, CTGF cross-reacted with PDGF antibody. Highly specific antibodies to PDGF A or PDGF B were used in order to determine that the highly expressed protein after TGF-β stimulation was CTGF, and not PDGF. Moreover, this showed that CTGF functioned as the autocrine mediator of DNA

synthesis in TGF-β treated human newborn foreskin fibroblasts. The investigation of the expression of CTGF transcripts in various cell types such as human umbilical vein endothelial cells (HUVEC), human foreskin fibroblasts, TGF-β1 treated human foreskin fibroblasts, human peripheral neutrophils and human peripheral blood monocytes, showed that CTGF expression is limited to TGF-β1 stimulated cells, and to the endothelial and fibroblastic cells during wound repair. This article is a strong verification of the biological presence of CTGF, and partial CTGF stimulation by TGF-β (Igarashi et al. 1993). CTGF stimulation by TGF-β was further evaluated by the same group of investigators, in which Northern blot and run-on transcription experiments showed evidence of the direct TGF-β activation of CTGF gene transcription. The experimental protocol consisted of the linkage of the 5' flanking region of human CTGF gene fragments to luciferase reporter gene constructs. The evaluation of luciferase activity was used as a tool to investigate the direct TGF-β activation of CTGF gene transcription. TGF-β induced 30 fold increase in luciferase activity in NIH/3T3 fibroblasts compared to untreated cells after 24 hours of incubation. PDGF and FGF induced 2-3-fold increases. Moreover, the stimulation of different cell lines by TGF-β to produce increased levels of CTGF occurred in a cell type specific manner. Thus, TGF-β stimulated CTGF production by human skin fibroblasts, fetal bovine aortic smooth muscle cells, and NIH/3T3 fibroblasts but not by epithelial cell lines. More interestingly, this study demonstrated that a 13-nucleotide sequence for a novel TGF-beta cis-regulatory element was also the CTGF promoter sequence. This proved the presence of an important TGF-β regulatory element on the CTGF promoter sequence. Furthermore, TGF-β was not able to induce CTGF transcription in deletion mutants or point mutations in the region of the CTGF promoter sequence. These studies explored possible mechanisms by which TGF-β stimulated CTGF production, and presented important data regarding identification of specific cell types

that produce CTGF, and identification of functional transcriptional mechanisms (Grotendorst *et al.,* 1996). Whether CTGF has mitogenic activity or not has great importance, since it is clear that it plays an important role as the downstream mediator of TGF-β. Normal rat kidney fibroblasts (NRK) fibroblasts were cultured in order to evaluate the mitogenic activity of CTGF. CTGF (20-50 ng) caused up to a six fold dose dependent increase of mitogenic activity of NRK fibroblasts. Interestingly, in these experiments, the investigators could not find any synergistic mitogenic activity between TGF-β and CTGF. Comparison of the effects of CTGF with TGF-β on the regulation of type I collagen, integrin, and fibronectin mRNA levels was evaluated in cultured NRK fibroblasts. Recombinant CTGF and TGF-β (10 ng/ml) added to NRK fibroblasts then incubated for 24 hours. Northern blot analysis of integrin mRNA levels, immunoprecipitation experiments for fibronectin, and analysis of pepsin digested collagen were performed. Northern blot analysis of integrin mRNA levels showed a three fold increase by TGF-β, and a 15 fold increase by CTGF. More importantly, the immunoprecipitation experiments for fibronectin and SDS-PAGE of pepsin digested collagen showed a 2.5 fold stimulation of collagen synthesis and a 3 fold increase in fibronectin synthesis by TGF-β and a 5 fold stimulation of collagen and fibronectin synthesis by CTGF (Frazier *et al.,*1996).

Studies have investigated whether additional factors or agents stimulate CTGF production. In that context, i*n vitro* stimulation of CTGF by dexamethasone but not TGF-β was shown in cells. Even though TGF-β stimulation of CTGF has been emphasized in the literature, dexamethasone stimulation of CTGF is of great interest because CTGF may have more wide spread functions than being a down stream mediator of TGF-β. In these experiments, fibroblasts were cultured with or without 1 μM of dexamethasone. CTGF expression was stimulated by dexamethasone in a time (1-10 hr). Addition of 1 ,10 and 100 nM of

dexamethasone stimulated CTGF 0, 8 and 14 fold, respectively. The authors suggested that there was no stimulation of CTGF by TGF-β, since its expression may be down regulated by dexamethasone (Dammeier *et al.*,1998).

The Detection of CTGF in Abnormal Tissues

The clinical appearance of phenytoin induced gingival overgrowth is fibrotic, showing excessive stippling and firm appearance. Interestingly, several articles suggest that CTGF might be involved in fibrotic skin disorders such as skin sclerosis. Skin fibroblasts generated from 5 healthy control patients and 15 scleroderma patients were cultured and stimulated by TGF-β then fixed and subjected to *in situ* hybridization, to detect CTGF mRNA. CTGF mRNA was not present in normal skin tissues whereas 12 of 15 scleroderma samples showed the presence of both CTGF and sclerosis. Moreover, fibroblasts expressing CTGF were more abundant in tissue samples at the sclerotic stage than at the inflammatory stage and no CTGF mRNA expression was found in the samples from the atrophic stage (Igarashi *et al.*,1995). The same group of investigators showed similar patterns in localized scleroderma, kelloid, and other fibrotic skin disorders (Igarashi *et al.*, 1996). Indeed CTGF mRNA appears to be expressed whenever there is fibrosis in different tissues or organs such as in renal fibrosis (Ito *et al.*, 1998) as well as bleomycin-induced lung fibrosis (Lasky *et al.*, 1998) and liver fibrogenesis (Paradis *et al.*,1999). Moreover CTGF is also likely to be involved in atherosclerosis. The isolation of a cDNA clone from human aorta showed that it was identical to CTGF. The expression of CTGF mRNA levels was much higher in atherosclerotic blood vessels compared to normal arteries. TGF-β stimulated

CTGF in vascular smooth muscle cells, and high levels of both CTGF mRNA and protein were expressed in advanced atherosclerotic lesions of human arteries (Oemar *et al.*,1997).

Furthermore, frozen sections taken from 11 human mammary carcinomas as well as separate mouse models were obtained and subjected to RNA extraction and Northern blotting, and *in situ* hybridization for CTGF and TGF-β probes. Despite the absence of human control mammary samples and low sample number, it was also suggested that TGF-β1 and CTGF might have roles in cancer stroma formation as a consequence of wound repair. (Frazier and Grotendorst, 1997). In this aspect, CTGF may be used as a marker for malignancy. CTGF gene expression was thought to be important to differentiate between benign and malignant mesenchymal tumors (Igarashi *et al.,* 1998). In addition, high levels of CTGF transcripts were expressed in human pancreatic cancer tissues (Wenger *et al.*, 1999).

The Presence of CTGF in Various Types of Normal Tissues

CTGF was detected in a chondrocytic cell line (Nakanishi *et al.,* 1997) and in the rat central nervous system (Kondo *et al.,* 1999). A novel connective tissue growth factor-like (CTGF-L) cDNA encoding cells a 250-amino acid single chain polypeptide was also identified and cloned from primary human osteoblasts (Kumar *et.al.*,1999).

Transforming Growth Factor Beta

The Discovery and the Structure of Transforming Growth Factor Beta

TGF-β1 is a fibrogenic factor that stimulates extracellular matrix accumulation in different tissues. Initially, two types of transforming growth factors were isolated from murine sarcoma virus- transformed 3T3 cells. The one competing with epidermal growth factor (EGF) was named TGF-β, and the other one not competing with epidermal growth factor (EGF) was named TGF–α. TGF-β was only able to stimulate the formation of large cell colonies in soft agar with the synergy of TGF–α or EGF (Anzano *et al.,* 1982). TGF-β was then isolated and purified from human placenta (Frolik *et al.,* 1983), and TGF-β was found to be a 25 kDa protein consisting of two subunits of 12.5 kDa (Assoian *et al.,* 1983). Functionally, TGF-β was also shown to accelerate experimental wound healing in rats (Roberts *et al.,* 1983). There are three known mammalian TGF-β genes which are all homologues, TGF-β1, TGF-β2 (Cheifetz *et al.*,1987) and TGF-β3 (Kiritsy *et al.,* 1993). TGF-β's are also important immune system modulators, since TGF-β1 and TGF-β2 were shown to inhibit the proliferation and function of several types of immune cells (Sing *et al.*,1988).

Effects of Transforming Growth Factor Beta on Extracellular Matrix

TGF-β1 is known to stimulate collagen production and mature insoluble collagen accumulation by fibroblasts, osteoblasts and keratinocytes (Centrella *et al.*, 1992; Meisler *et al.*, 1997). Extracellular enzymes that post-translationally modify procollagen in the formation of a mature insoluble collagenous extracellular matrix have been shown to be up-regulated in fibroblasts and osteoblasts by TGF-β1. For example, procollagen C-proteinase (Lee *et al.*, 1997), and the extracellular enzyme lysyl oxidase (Boak *et al.*, 1994; Feres-Filho *et al.*, 1995; Shanley *et al.*, 1997) are up-regulated by TGF-β1 in a variety of fibrogenic cell types.

Lysyl Oxidase

Lysyl oxidase catalyzes the oxidative deamination of peptidyl-lysine in elastin precursors, and lysine and hydroxylysine residues in collagen, to form peptidyl-α-aminoadipic-δ-semialdehyde and peptidyl-δ-hydroxy-α-aminoadicpic-δ-semialdehyde residues, respectively. These aldehydes then undergo non-enzymatic reactions resulting in the cross-linkages known to be critical in the formation of mature insoluble elastin and collagens (Kagan 1986; Kagan and Trackman 1991). Lysyl oxidase is a copper-dependent enzyme, and is specifically inhibited by β-aminopropionitrile (BAPN). Connective tissue abnormalities known as lathyrism resulting from *in vivo* inhibition of lysyl oxidase activity by either BAPN-feeding or feeding animals a copper deficient diet have been described (Rucker *et al.,* 1975). Abnormalities include malformed and weak bones, as well as increased development of hernias (Selye 1957). Lysyl oxidase is synthesized as a 50 kDa glycoprotein and is processed extracellularly to produce the 30 kDa molecular form known to be active (Trackman *et al.,*1992). Since lysyl oxidase control one of the most important steps in collagen biosynthesis, and since it is an enzyme functioning extracellularly , it may well be the main target of CTGF in order to stimulate collagen accumulation. Mechanisms of stimulation, if they occur, could consist of CTGF being the downstream mediator of TGF-β1, or by CTGF itself triggering the stimulation of lysyl oxidase biosynthesis or activation.

Collagenase

It was suggested that gingival overgrowth might result from increased ground substance caused by decreased extracellular matrix breakdown due to the decreased amount of collagenase (Hall *et al.,* 1982). In order to evaluate this theory, cell cultures were generated from biopsy samples from the interdental papilla of six patients taking phenytoin and three control patients. Then the conditioned media were proteolytically pre-activated under serum free conditions, and collagenase activity was measured by using ^{14}C-glycine labeled collagen fibril gels (Bauer et *al.,* 1972). No statistically significant collagenase activity secreted into serum-free medium was found in human gingival fibroblasts taken from healthy patients or from patients taking phenytoin. Total immunoreactive collagenase per cell culture was defined in the normal and phenytoin patients' fibroblasts. Certain cell cultures showed high levels of active, and inactive forms of collagenase, but collagenase levels could not be related to phenytoin effects. In general, the collagenase activity decreased with time as the cell cultures became more confluent. Even though the cell lines showed variations in collagenase activity, only released collagenase was measured and cell layer was not assayed for a more definitive conclusion (Hassell 1982). More recent studies showed that phenytoin and cyclosporin A were able to suppress MMP-1 and TIMP-1. Human gingival fibroblasts were cultured with or without 20 mg/ml of phenytoin or 200 ng/ml of cyclosporin A. RT-PCR analyses were done after harvesting total RNA and cellular proteins and lysosomal proteolytic activity was also determined. In addition, the investigators used immunohistochemistry methods in order to detect lysosomal enzymes in overgrown gingiva

samples from patients taking phenytoin. The results of the RT-PCR analyses showed that both phenytoin and cyclosporin A suppressed the expression of MMP-1, TIMP-1, and cathepsin A, but not cathepsin B in a time-dependent manner. Northern blot analysis showed that the inhibition of enzyme expression was stronger on human gingival fibroblasts treated with phenytoin then with cyclosporin A (Yamada *et al.,* 2000). Based on these studies, it might be important to understand whether CTGF is an important inhibitor of collagenase or that inhibition works through another mechanism independent of CTGF.

Immunohistochemistry methods were also used in order to correlate collagenase to drug induced gingival overgrowth. Specimens from control and drug induced gingival overgrowth patients were taken and matrix metalloproteinases, MMP-1 (collagenase) and MMP-3 (stromelysin) were localized on the sections. MMP-1 positive staining was seen in the majority of fibroblasts in the connective tissue of the control samples, where the positive staining for MMP-1 in overgrown tissue samples was only present in a small number of fibroblasts (Thomason *et al.,* 1998).

Our interest in collagenase centers on whether this enzyme is actively involved in a CTGF related mechanism, and the studies cited above indicate that there may be a relationship between CTGF and collagenase.

BMP-1, Tolloid and Tolloid Like proteins

The Discovery and the Distinction of Bmp-1, Tolloid and Tolloid Like Proteins

The identity of Procollagen C-proteinase (PCP) and bone morphogenetic protein-1 (BMP-1) is one of the most important discoveries about BMP-1 gene family members (Kessler *et al.,* 1996). Bone morphogenetic protein-1 (BMP-1) is a metalloproteinase that is the C-propeptidase of procollagen types I, II, and III. Unlike the other bone morphogenetic proteins, BMP-1 is not a transforming growth factor-beta (TGF-β) superfamily member, but it is the prototype of a family of proteinases implicated in pattern formation during development in different organisms (Takahara *et al.,* 1994). Even though BMP-1 itself is not a TGF-β superfamily member, two of the TGF-β super family members; BMP-2 and BMP-4 show sequence similarities to BMP-1 (Shimell *et al.,* 1991). BMP-1 may also induce TGF-β-related genes during embryogenesis (Fukagawa *et al.,*1994).

The structural similarity between tolloid and BMP-1 was first noticed after the cloning of the tolloid locus in Drosophilla. Tolloid has an N terminal region similar to astacin metalloproteinases, two EGF-like repeats, and five copies of a repeat found in human complement proteins C1r and C1s. It was found that the *Drosophila* tolloid sequence is 41% identical to human bone morphogenetic protein 1 (Shimell *et al.,* 1991). Tolloid itself is in the same gene family as Decapentaplegic (dpp) which is a important for dorsal patterning of embryos (Ferguson *et al.,* 1992). The isolation of a BMP-1 related gene in mice showed that

there may be other gene products resembling either tolloid or BMP-1. This finding is important because BMP-1 and Tolloid may have its distinct functions (Fukagawa *et al.,* 1994).

Tolloid and its related forms may have evolved independently and may acquire distinct, tissue specific roles in *Drosophila* development (Nguyen *et al.,* 1994). Human, and mouse mammalian tolloid (mTLD) is the alternatively spliced form of the *Bmp1* gene, and is a longer protein with a domain structure identical to that of Drosophila Tolloid. A third form was also detected in *Drosophila* tissue samples. Each of these forms has been located in different tissues and some, such as BMP-1, is not present in the brain at all. These findings show that different of the same gene products may have distinct functions (Takahara *et al.,* 1994). The same group described the organization of the 46-kb, 22-exon human *Bmp1/mTld* gene that encodes alternatively spliced forms of the gene (Takahara *et. al.,* 1995). Furthermore, the *Bmp1* gene has a third alternatively spliced isoform. This isoform is called bone morphogenetic protein 1/His. It is structurally shorter and has a modified C- terminus. All three alternatively spliced forms of *Bmp1* gene are expressed in placenta but showed differences in their expression patterns in other soft tissues (Janitz *et al.,* 1998).

A novel mammalian gene with a domain structure identical to that of BMP1, mTLD was discovered and named as mammalian tolloid-like 1 (*mTll1*). Mammalian tolloid-like 1 (*mTll1*), has 76% identity with *bmp1* for amino acid residues including the proteinase domain. By contrast, the N-terminus domains showed little similarity. Mammalian tolloid-like (*mTll)* shows additional strong expression in structures of the developing, neonatal, and adult brain where the expression of *Bmp1* gene and *mTld* gene has not been observed (Takahara *et al.,* 1996). The tolloid-like genes were named as tolloid-like 1 (*mTll1*) after the discovery of mammalian tolloid-like 2 (*mTll2*). *mTll1* is essential for the formation of the mammalian heart and interventricular

septum (Clark *et al.,* 1999). Mammalian tolloid-like 2 (*mTll2*) has high sequence similarities to *mTll1* (Scott *et al.,* 1999).

Functional Differences of BMP-1, Tolloid and Tolloid Like Proteins

Transgenic animals null for the *Bmp1* gene, were generated by homologous recombination, and deletion of DNA sequences encoding the active site of the protease domain common to all of the spliced forms of the *Bmp1* gene. Homozygous mutant embryos had a normal skeleton, but had herniation of the gut in the umbilical region. The homozygous animals did not survive beyond birth. The extracellular matrix of the amnion contained collagen fibrils with an abnormal morphology, and contained partially processed procollagen molecules determined by electron microscopy (Suzuki *et al.,* 1996).

BMP-1 processes lysyl oxidase and laminin 5. Moreover, BMP-1 is up-regulated by TGF-β1. The secreted form of BMP-1 has to be processed to an active form, but this activity is cell type-dependent. Procollagen C-proteinase enhancer (PCPE) is a glycoprotein that stimulates the procollagen C-proteinase activity of BMP-1. The lack of PCPE in keratinocytes left BMP-1 and mTld as unprocessed pro-enzymes. Moreover, even stimulation by TGF-β1 does not replace the absence of PCPE (Lee *et al.,* 1997) .

BMP-1 gene products also pattern ventral tissues of the zebra fish and Drosophilla embryo while BMP-1 inhibitors, such as chordin, noggin, and follistatin, are implicated in dorsal mesodermal and neural development. A BMP-1 activity gradient occurs at the end of gastrulation, when the ventralmost marginal cells of the embryo are close to the dorsal chordin-expressing cells. At this time, chordin may diffuse to the most ventral regions and inhibit high BMP-4 activity levels. Interestingly, when mTld is present, chordin activity would be inhibited

by the proteolytic cleavage of chordin by mTld . This inactivation of chordin leads to the increased BMP-4 activity. This appears to be a inhibitory mechanism for BMP-4 activity (Connors *et al.,* 1999). Chordin was also found to be a BMP-4 antagonist in zebra fish where *mTld* also antagonizes chordin activity. Tolloid activates BMP-4 by proteolytically cleaving and inactivating chordin (Blader *et al.,* 1997). BMP-1, mTLD, mTLL-1, and mTLL-2 have differences in the processing ability of the fibrillar collagen precursors and in the cleavage of chordin. mTLL-1 is able to specifically process procollagen C-propeptides at the right site same as BMP-1, and mTLD, while mTLL-2 is not. In terms of distinguishing the differences among the *BMP-1 gene* family members, BMP-1 and mTLL-1 are able to cleave chordin, at sites similar to procollagen C-propeptide cleavage sites while mTLD does not cleave chordin. Despite the differences between the family members, BMP-1 is the major chordin antagonist in early mammalian embryogenesis and in pre- and postnatal skeletogenesis. Indeed our focus on BMP-1, m-TLD, mTLL-1, and mTLL-2 is to determine whether these enzymes are able to cleave pro-lysyl oxidase with the same efficiency or whether they may have differences in cleavage activity as it is the case for chordin cleavage (Scott *et al.,* 1999).

MATERIALS AND METHODS

Part One

Reagents.

Human recombinant TGF-β1 was purchased from Austral Biologics (San Ramon, CA). Human recombinant CTGF was provided by FibroGen Corporation (South San Francisco, CA) and was produced using a baculovirus expression system. Affinity purified rabbit anti-hrCTGF (pAb2) and preimmune IgG was generously provided by Fibrogen Corporation. All other reagents were purchased from Sigma/Aldrich unless otherwise indicated.

Preparation of Fibroblastic Cultures

Non-hyperplastic gingival tissue from different healthy adult subjects undergoing routine periodontal procedures were obtained from the Boston University School of Dental Medicine Department of Periodontology Clinic after informed consent according to Boston University Institutional Review Board approved procedures. Fibroblastic cells were obtained from explants (Piche *et al.*, 1989). Connective tissue was surgically separated from the epithelium, and the connective tissue was diced into 2 - 3 mm^3 pieces. Tissues were placed in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-treated new born calf serum (NBS) plus 0.1 mM nonessential amino acids and antibiotics in a 100 mm tissue culture plate and cultured at 37° C in a 5% CO_2 atmosphere. Medium was changed weekly until cells were confluent. Cells were then dissociated with trypsin/EDTA and cultured in five new plates and grown to confluence. Cells

were then frozen in aliquots containing 500,000 cells/ml and were designated passage 3 when re-cultured. Cell cultures are designated as follows: N5 and N6 and N7 cells were cultured from tissues obtained from 29, 30, and 32 year old females, respectively, N4 cells were obtained from a 35 year old male. NHCT 8, NHCT 11 and NHCT 12 were obtained from a female donor age 30, and from male donors age 42 and 43, respectively.

Cell culture. Human gingival fibroblasts at passage 3 from frozen stocks were cultured at 37 °C in a 5% CO_2 atmosphere in DMEM containing 10% NBS, 0.1 mM non-essential amino acids and antibiotics in 100 mm tissue culture plates. Cultures were re-fed with fresh medium twice per week. When confluent, cells were passaged at a ratio of 1:7, cultured to confluence, and passaged again at a ratio of 1:7. Cells were grown for three days to about 75 - 85% confluence, and were placed in serum-free DMEM containing 0.1% bovine serum albumin, 0.1 mM non-essential amino acids and antibiotics and cultured for an additional 24 hours. Cells were then re-fed with this medium containing either the test cytokine or vehicle. Cells were harvested at intervals as described below. Cells were utilized only up to passage 7 in order to minimize the possibility of phenotypic variability that might occur after additional passages.

Lysyl Oxidase Enzyme Activity

Lysyl oxidase enzyme activity was determined utilizing a tritium release assay with human tritiated recombinant tropoelastin as substrate (Bedell-Hogan *et al.*, 1993). Aliquots of media samples (0.5 ml) were incubated in the presence and absence of the lysyl oxidase inhibitor (0.5 mM β-aminopropionitrile) in a final volume of 1 ml of 0.1 M borate, 0.15 M NaCl pH 8.0 and 160,000 cpm of tropoelastin at 55° C for 60 min. Released tritiated water was recovered by vacuum distillation, and quantitated by liquid scintillation spectrometry. Lysyl oxidase activity is

defined as tritium released above reactions containing β-aminopropionitrile. Enzyme activity determinations were normalized to the number of cells counted from the same cultures utilizing a hemocytometer. Reactions were performed in quadruplicate to permit analyses of the statistical significance of differences in enzyme activity between cytokine treated cultures and untreated control cultures by unpaired *t*-test. Differences were considered significant when $p < 0.05$. Data are generally presented as fold-changes compared to controls.

Isolation and analysis of RNA

Total RNA was isolated from three 100 mm culture dishes utilizing TRIZOL Reagent (Gibco/BRL) (Chomczynski, 1993). Aliquots of RNA (10 μg) were subjected to 1% agarose/formaldehyde gel electrophoresis followed by Northern blotting. Blots were analyzed with radiolabeled cDNA probes utilizing random primer methodology for lysyl oxidase (Mariani *et al.*, 1992), α-1-type I collagen (Genovese *et al.*, 1984) and human connective tissue growth factor (CTGF) provided by Fibrogen Corp., South San Francisco, CA, Collagenase (hMMP-1), and with a DNA probe for 18S rRNA (Hillis and Dixon, 1991). Signals were quantitated by laser scanning densitometry, and values were normalized using the signal obtained for 18S rRNA.

Western Blotting Analyses

Human gingival fibroblast cultures were treated with or without TGF-β1 as specified in serum-free media containing 0.001% BSA. At each harvest, conditioned media from two 100 mm plates were pooled, and proteins precipitated by the addition of 1/9 volume of 100% trichloroacetic acid at 4° C for 18 hours. Precipitated proteins were collected by centrifugation, and washed twice with 1 ml ice cold acetone to remove residual trichloroacetic acid. The pellet was then air dried and dissolved in 0.5 ml SDS PAGE sample buffer. Similarly, cell layers from the same plates were directly extracted into a total volume of 0.5 ml SDS PAGE sample buffer. Samples were then boiled for five minutes and fifty microliter aliquots were subjected to SDS PAGE and Western blotting (Towbin *et al.*, 1979). Each 50 μ l loading is equivalent to 10 per cent of the media or cell layer protein produced by two 100 mm cell culture plates. Typically each plate contained 1.5 – 2.0 x 10^6 cells. Cell number per plate comparing different plates cultured at the same time did not vary by more than 20% either in the presence or absence of TGF-β1. Affinity purified rabbit anti-CTGF antibody (pAb2) or pre-immune IgG were used at a concentration of 0.1 mg/ml. Signals were detected with goat anti-rabbit IgG coupled to alkaline phosphatase, and Western Blue detection reagent (Promega).

Collagen Accumulation

Human gingival fibroblasts at passage seven were cultured to confluence in DMEM containing 10% NBS, and 1 mM non-essential amino acids and antibiotics in 100 mm cell culture dishes. Cells were then cultured in 15 ml of this same medium containing 50 μg/ml ascorbate either in the presence or absence of 50 ng/ml CTGF. Cells were re-fed fresh medium containing ascorbate +/- 50 ng/ml CTGF once every 48 hours for the duration of the experiment.

Cells were harvested after 0, 4, 10, and 18 days as follows. Cell layers were rinsed three times with phosphate buffered saline, and were then scraped and suspended in 5 ml of 0.01% Triton-X 100. Aliquots of this suspension were analyzed for DNA content (Vytasek, 1982). Additional aliquots were hydrolyzed in 6N HCl at 110° C for 22 hours, and then subjected to amino acid analysis. Collagen content was calculated based on the hydroxyproline content of 13% by weight (Franceschi *et al.*, 1994).

Sampling of Gingival Tissues

Gingival tissue samples were obtained from patients undergoing periodontal surgery in the Department of Periodontology and Oral Biology of Boston University, the Franciscan Children's Hospital and Rehabilitation Center, the Department of Pediatric Dentistry of Boston University, and the Department of Periodontology of University of Istanbul. Samples from 27 patients were included in the study. Classification of these samples was as follows: 1) phenytoin-induced gingival overgrowth (PHE, n=9), 2) nifedipine-induced gingival overgrowth (NIF, n=4), 3) cyclosporin-A-induced gingival overgrowth (CSA, n=5), and 4) control tissues (C, n=9) from systemically healthy donors. Consent from the patients was obtained before the study. The consent forms were approved, respectively, by the Institutional Review Board of Boston University Medical Center, the Institutional Review Board of the Franciscan Children's Hospital and Rehabilitation Center, and by the Department of Periodontology of the University of Istanbul. All patients in this study were 20 years of age or older. For CSA patients, at least 6 months with no rejection of the renal grafts had passed after the transplantation, and no patients received multiple transplantation. Gingival biopsies were obtained from the attached gingiva of the anterior buccal, mesial, or distal aspects of the teeth during gingivectomy (Groups 1-3) and flap surgery for pocket elimination or preprosthetic crown lengthening procedure for the control

group (Group 4). The teeth neighboring the gingival overgrowth were free from endodontic and orthodontic complications. None of the patients had any restorations, crowns or bridge constructions at their dentition around the gingival overgrowth and they did not show any sign of clinical attachment loss.

Immunohistochemistry of Overgrown Human Gingival Samples

Tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C for 4 hours and were incubated in 30% sucrose overnight. They were then placed in 2-methylbutane at –80° C. At least 20 serial 6 μm frozen sections were made for each tissue sample on a cryostat. Immunohistochemistry was carried out using a temperature controlled staining system in order to standardize the staining conditions[1]. Primary antibodies used for immunostaining were affinity purified rabbit polyclonal antibodies against CTGF (pAb2) and pre-immune IgG (pCAb2) generously provided by FibroGen Corporation[2], affinity purified polyclonal TGF-β1 antibody #sc-146. Working concentrations for primary antibodies were 8 μg/ml for CTGF and 3μg/ml for TGF-β1. Serial sections were routinely stained with pre-immune IgG or non-immune rabbit IgG and served as negative controls. After application of primary antibodies, sections were washed in PBS + 0.01% TWEEN 20, and treated with 0.3% H_2O_2 in NaN_3 methanol to suppress endogenous peroxidase activity. Visualization of primary antibody binding was achieved using avidin-biotin-horseradish peroxidase complex employing diaminobenzidine as the chromogen and Elite Vecta Stain kits[3]. Sections were counter-stained with hematoxylin.

---

[1] Fisher Biotech, Microprobe System, Fisher Scientific, Pittsburgh, PA
[2] FibroGen Corporation, South San Francisco, CA
[3] Vector Laboratories, Burlington, CA

Evaluation of the Immunohistochemistry of the Human Overgrown Gingival Tissue Section Samples

The orientation of each sample and identification of tissue sites were determined at 100X and 400X magnification, respectively. Five sites with corresponding areas of 0.09 mm^2 were defined and utilized for quantitative analyses of immunohistochemical staining in all tissue samples. Quantitation was done by computer-assisted image-analysis[4] and data were collected from 3 – 5 serial sections per tissue specimen per assay. The tissue sites, as shown schematically in Figure 1 , encompassed the following areas:

1. Oral-Sulcular Epithelium and Subepithelial Connective Tissue
2. Oral Epithelium and Subepithelial Connective Tissue
3. Oral Epithelium and Subepithelial Connective Tissue
4. Sulcular Epithelium and Subepithelial Connective Tissue
5. Middle-Deep Connective Tissue

Immunostaining for intracellular CTGF was evaluated by counting the number of stained cells per unit area. In addition, analysis of extracellular staining of CTGF, intra- and extracellular staining of TGF-β1 was accomplished by grading the intensity of staining between 0 and 4. This grading was done as follows:

Grade 0: No staining

Grade 1: Staining in less than ¼ of the total inspection area

Grade 2: Staining in more than ¼ but less than ½ of the total inspection area

Grade 3: Staining in more than ½ but less than ¾ of the total inspection area

[4] Image-Pro Plus 4.0, Media Cybernetics, Silver Spring, MD.

Grade 4: Staining in more than ¾ of the total inspection area

Corresponding pre-immune (for CTGF) or non-immune (for TGF-ß1) stained slides from serial sections were used as controls in order to determine background staining which was low or negligible. The same grading system was used to analyze the degree of fibrosis measuring the abundance and density of fibroblasts and collagen-like connective tissue fibers in hematoxylin stained sections. The number of inflammatory cells per 0.09 mm^2 unit area was also determined from hematoxylin stained sections to assess the relative degree of inflammation. Two investigators utilizing coded samples with excellent agreement made quantitative measurements and assessments of histologic samples independently. Results are expressed as mean ± standard deviation for each evaluated site. Data were collected from 3 – 5 serial tissue sections per assay per specimen. For statistical analysis, the Mann-Whitney test was used for the comparison of differences. Significance was defined as $p<0.05$.

Statistical analysis

Results are expressed as mean ± st.dev for each evaluated site. For statistical analysis, the Mann-Whitney test was used for the comparison of differences and the Spearman rank correlation test was used for the analysis of relationships between the variables. Significance was defined as $p<0.05$.

MATERIALS AND METHODS

Part two

The maltose binding protein/lysyl oxidase fusion protein

The maltose binding protein/lysyl oxidase fusion protein was produced and purified as previously described (Feres-Filho *et al.,* 1995). Polyclonal rabbit anti-maltose binding protein antibodies were purchased from New England Biolabs (Beverly, MA), and rabbit anti-bovine lysyl oxidase antibodies were affinity purified utilizing immobilized lysyl oxidase fusion protein. Affinity purification of anti-lysyl oxidase was accomplished utilizing recombinant rat lysyl oxidase fusion protein covalently attached to Sulfolink (Pierce), and specific antibodies were purified according to the manufacturer's protocol. Titer of the purified lysyl oxidase antibody was determined by Western blotting against recombinant rat lysyl oxidase. Alkaline phosphatase-coupled goat anti-rabbit antibodies and Western Blue detection reagents were purchased from Promega.

Pro-lysyl Oxidase Processing Assays

Maltose binding protein/lysyl oxidase fusion protein (7 μg) was incubated with 30 ng of recombinant mBMP-1, mTLD, mTLL-1, or mTLL-2 for either 45 min or 4 hours at 37^{o} C in a final volume of 200 μl; 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5. Reactions all contained 40 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 0.4 mM PMSF to inhibit non-specific proteinases. Reactions were stopped by adding an equal volume of SDS PAGE sample buffer, and boiling for three minutes. 50 μl aliquots were subjected to 10% PAGE and Western blotting (Towbin *et al.,* 1979) to PVDF membranes (NEN Products), and duplicate blots were blocked with 1% bovine serum albumin in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.05%

Tween 20. Blots were incubated with anti-maltose binding protein antibody or affinity purified anti-lysyl oxidase antibody at a dilution of 1:10,000 at room temperature overnight. Blots were washed and then incubated with alkaline phosphatase goat anti-rabbit IgG according to the manufacturer. Bands were detected using the stabilized nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate Western Blue reagent (Promega).

Recombinant Enzymes

Recombinant human BMP-1, mTLD, mTLL-1, and mTLL-2 were produced as C-terminal Flag-tagged proteins in transfected 293-EBNA cells, and purified as previously described (Scott *et al.,* 1999), and were provided by Dr. Daniel Greenspan. Activity of each enzyme against known procollagen and Chordin substrates was verified before initiating experiments with pro-lysyl oxidase.

RESULTS

Part one

Regulation of CTGF by TGF-β1

Regulation of collagen and lysyl oxidase by TGF-β1(Hong et al.1999) is slow compared to previous studies performed with murine osteoblastic cells (Feres-Filho *et al.*, 1995). As noted, CTGF has been proposed to mediate effects of TGF-β1 on extracellular matrix accumulation in some cell types (Frazier *et al.*, 1996). of Thus, TGF-β1regulation of CTGF was investigated in order to evaluate the possibility that CTGF might partially or fully mediate the fibrogenic effects of TGF-β in human gingival fibroblasts. Time-dependent regulation of CTGF mRNA levels by 400 pM TGF-β1 in N-5 human gingival fibroblasts was determined. CTGF mRNA levels increased dramatically from nearly undetectable levels in untreated controls, to easily detectable levels after four, 24, and 48 hours of treatment. Scanning laser densitometry indicates that the stimulation of CTGF mRNA levels was at least 20-fold, and was maximally stimulated at 24 hours of treatment. CTGF was not induced by IL-6 or by bFGF in these cells. TGF-β1-dose dependent increases in CTGF mRNA levels were found at 48 hours of treatment (Hong *et al.*,1999). Consistent with studies performed in human glioma cells (Xin *et al.*, 1996), minor high molecular weight CTGF transcripts of about 3 and 7 kb were sometimes observed in RNA isolated from TGF-β1 treated human gingival fibroblasts (Hong *et al.*,1999).

In the present studies CTGF protein levels were determined in the media and cell layers of TGF-β1 treated fibroblasts by Western blotting analysis utilizing an affinity-purified polyclonal antibody (Figure 2). Cultures treated with 400 pM TGF-β1 for 4, 24 and 48 hours all had increased levels of 42 kDa CTGF protein in the cell layer compared to untreated control cell

cultures. Interestingly, in the medium, a time-dependent increase in CTGF immunoreactive bands was observed in both CTGF and control media, but clearly more CTGF-related proteins were found in the media from TGF-β1 treated cells. At 24 and 48 hours of treatment, molecular species of 38 - 42 kDa and approximately 20 kDa were clearly apparent in the media, but only 38 - 42 kDa molecular forms CTGF were present in the cell layer. Pre-immune IgG resulted in no staining (Figure 2, lane P), and demonstrate the specificity of the affinity-purified CTGF antibody. These results establish that CTGF is induced in human gingival fibroblasts by TGF-β1, and are consistent with the notion that extracellular proteolytic processing of a CTGF precursor occurs.

Regulation by Exogenous CTGF

Next, we evaluated whether CTGF exogenously added to human gingival fibroblasts regulated lysyl oxidase enzyme activity, and mRNA levels of lysyl oxidase and α-1 type I collagen. In these experiments 0, 1 ng/ml, 10 ng/ml and 50 ng/ml CTGF was added to human gingival fibroblasts in serum-free medium, and lysyl oxidase enzyme activity in media samples was determined after 24 and 48 hours normalized to cell number. Data from two experiments are presented in Figure 3, and show 1.3-fold and 1.7-fold stimulation of lysyl oxidase enzyme activity by 50 ng/ml CTGF at 24 and 48 hours, respectively. Lower levels of CTGF were weaker stimulators of lysyl oxidase enzyme activity. Northern analyses from both experiments indicated no increases in lysyl oxidase and collagen mRNA levels at 4 and 24 hours, and increases at 48 hours not exceeding 1.2-fold compared to untreated control levels (data not shown). Stimulation

of lysyl oxidase enzyme activity by 48 hour 50 ng/ml CTGF treatment of gingival fibroblasts cultured from six different subjects was evaluated to determine whether this effect was unique to one subject. Data pooled from quadruplicate assays of media samples from cells cultured independently from each of six different subjects indicated a mean 1.3-fold stimulation by CTGF of lysyl oxidase enzyme activity compared to untreated control cultures ($p<0.001$, unpaired *t*-test). All assays were normalized to cell number. Taken together, these results indicate that CTGF is a weak stimulator of lysyl oxidase enzyme activity, and does not stimulate lysyl oxidase or collagen mRNA levels in human gingival fibroblasts. Because CTGF regulation of gingival fibroblast lysyl oxidase and collagen is weak, and regulation by TGF-β1 is strong, it appears that CTGF may only partially mediate TGF-β1regulation of these products.

CTGF-Induced Collagen Accumulation

It is possible that small increases in lysyl oxidase enzyme activity over time may be functionally important, and might result in increased insoluble collagen accumulation. Lysyl oxidase catalyzes the final enzymatic step required for cross-linking of collagens and elastin. These cross-links serve to stabilize collagenous extracellular matrices and contribute to the efficiency of accumulation of insoluble collagen. In addition, it is possible that CTGF promotes insoluble collagen accumulation by gingival fibroblasts by other unidentified mechanisms. Thus, confluent N5 cells were grown in the presence and absence of 50 ng/ml CTGF as described in "Materials and Methods", and cell layers were analyzed for total DNA, and for total protein, and hydroxyproline levels by amino acid analysis. At day zero there was little collagen accumulation in both the control and CTGF treated cultures, as expected for cultures, which have not yet

accumulated extracellular insoluble collagen. Cell layer collagen/DNA increased after 4, 11, and 18 days in control cells, as expected for cultures accumulating insoluble collagen. CTGF increased cell layer collagen by 1.5-, 2.0, and 1.5-fold on day 4, 11, and 18, respectively (Table 1) experiment was performed twice with similar results. These data support that CTGF stimulates insoluble mature collagen accumulation in longer-term cultures of gingival fibroblasts.

The Inhibition of Collagenase by CTGF

We evaluated whether CTGF, exogenously added to human gingival fibroblasts, regulated collagenase (MMP-1) mRNA levels. In this evaluation, 0, 1 ng/ml, 10 ng/ml and 50 ng/ml CTGF was added to human gingival fibroblasts in serum-free medium, total mRNA was isolated 24 and 48 hours later. The Northern blots were obtained, and analyzed with radiolabeled cDNA probes utilizing random primer methodology for collagenase (MMP-1), and normalized to 18S RNA. Data were generated by evaluating optical density of the radiographs with phosphor imager. Data from the experiments are presented in Figure 4 and quantitative analysis indicate 0.72 units, 0.79 units, 0.80 units, 0.75 units of optical density after the addition, 0, 1 ng/ml, 10 ng/ml and 50 ng/ml CTGF, respectively for the 48 hour time point. However, the quantitative analysis of optical density after the addition, 0, 1 ng/ml, 10 ng/ml and 50 ng/ml CTGF fort the 24 hour time point was not possible since the signals were very weak. Visual inspection of the results from the 24-hour samples did not show evidence of collagen regulation. These results indicate that CTGF had no inhibitory effect on collagenase mRNA levels in human gingival fibroblasts.

Analysis Of Connective Tissue Growth Factor In Drug Induced Gingival Overgrowth

Hematoxylin stained sections made from all gingival overgrowth tissue specimens reveal hyperplasia with dense, elongated, and thin rete pegs inserted in deep connective tissue. In the phenytoin group, these elongations are more obvious and abundant compared to other types of drug-induced gingival overgrowth and the normal controls. Fibrosis, as determined by the abundance of fibroblasts and fibers, is clearly higher in phenytoin samples. Semi-quantitative data shows that this difference is statistically significant at all tissue sites compared to the control samples. Similarly, phenytoin samples are significantly more fibrotic than nifedipine samples in sites 1 - 3 ($p<0.05$, Figure 5). All five different sites in phenytoin samples are equally fibrotic, as determined from statistical comparisons of data obtained from each individual site. The cyclosporin A group contains the least degree of fibrosis compared to the other drug-induced gingival overgrowth groups, and is not significantly elevated compared to control in any site. Although there is a trend toward more fibrosis in nifedipine samples compared to control samples these differences are not statistically significant ($p>0.05$).

The degree of inflammation, as assessed by the number of mononuclear and polymorphonuclear inflammatory cells per unit area, is highest in cyclosporin A samples compared to the other drug-induced gingival overgrowth tissues and the control group (Figure 6). Inflammation in cyclosporin A samples is significantly higher than in control samples in all five sites ($p<0.05$), and is significantly higher in sites 3 and 5 compared to nifedipine samples ($p<0.05$). Inflammation in nifedipine tissues is significantly higher compared to control samples in site 4 ($p<0.05$). In contrast, inflammation in the phenytoin samples is not significantly higher than control samples in any site. All groups have the highest concentration of inflammatory cells in the subsulcular connective tissue-epithelium interphase (Figure 1, and Figure 6).

The level of both extra- and intracellular CTGF is significantly higher ($p<0.05$) and obviously elevated in all tissue sites in phenytoin samples compared to controls and compared to other drug-induced gingival overgrowth samples (Figure 7a and 7b). As shown in representative micrographs, extracellular CTGF staining is much more obvious in phenytoin samples (Figure 8, Figure 9). Extracellular CTGF localization is seen in connective tissue stroma (Figure 8). Pre-immune CTGF staining for all groups was negative as expected (Figures 8). Extracellular CTGF in the phenytoin group is high in all sites (Figure 8, Figure 9). Cellular CTGF staining is higher than control obviously elevated in all tissue sites in phenytoin samples compared to controls and compared to other drug-induced gingival overgrowth samples (Figure 8). As shown in representative micrographs, extracellular CTGF staining is much more obvious in phenytoin samples (Figure 8). Extracellular CTGF localization is seen in connective tissue stroma, perivascular areas, and in the endothelial layer of the blood vessels (Figure 8). Pre-immune CTGF staining for all groups was negligible as expected (Figures 8). Extracellular CTGF in the phenytoin group is high in all sites (Figure 8). Cellular CTGF staining is higher than control in all sites, but cellular CTGF staining is the highest in the subsulcular layer of phenytoin tissues (Figure 7 site 4, $p<0.05$). nifedipine samples contain the second highest extra- and intracellular CTGF staining. The pattern in this group is very similar to that of phenytoin samples (Figures 8). The extracellular CTGF in nifedipine samples (Figure 10) is significantly higher in all sites compared to the control group, while the difference compared to cyclosporin A is significant in all sites except site 4 ($p<0.05$). Intracellular CTGF staining in nifedipine samples is significantly higher compared to control(Figure 11) and cyclosporin A(Figure 12) specimens at sites 4 and 5 ($p<0.05$). In cyclosporin A samples, both the intra- and extracellular CTGF staining is increased at site 4 compared to other sites (Figures 8), and extracellular CTGF staining is significantly

higher than control tissues at site 4 ($p<0.05$). The intracellular CTGF staining is not significantly different compared to control samples in any of the evaluated sites ($p>0.05$). Control tissues express low extracellular staining of CTGF and no significant differences between the five tissue sites were found ($p>0.05$). Similarly, the control samples show the lowest cellular CTGF distribution throughout all the areas studied and did not show any significant differences between the five sites assayed ($p>0.05$). In summary, CTGF staining is clearly and dramatically elevated in phenytoin gingival overgrowth tissues in all sites. Nifedipine overgrowth tissues contain modestly elevated CTGF staining in a similar fashion, whereas in cyclosporin A overgrowth samples CTGF levels are, in general, close to control levels.

phenytoin samples contain the highest TGF-β1 staining in all tissue areas (Figure 8, Figure 13). At site 4, the difference is statistically significant ($p<0.05$) compared to all other samples. At sites 1 and 5, the TGF-β staining is significantly higher than in C samples ($p<0.05$). The other variations among tissue samples and sites are not statistically significant ($p>0.05$). Thus, although phenytoin samples contain both the most CTGF and TGF-β staining compared to other samples, there is not a strict pattern of elevated co-expression of CTGF and TGF-β in all sites.

Results

Part Two

Recombinant maltose binding protein/lysyl oxidase fusion proteins have been created and used as substrate to assess pro-lysyl oxidase proteolytic processing activity (Panchenko et al. 1996, and Feres-Filho et al. 1995). As shown in Figure 14, the amino-terminal end of the intact fusion protein consists of the maltose binding protein, and the carboxyl-terminal end is the rat lysyl oxidase pro-enzyme. Following proteolytic processing by procollagen C-proteinase, two products are generated: 30 kDa mature lysyl oxidase, and a 56 kDa maltose binding protein/lysyl oxidase propeptide product. Immuno blotting of products utilizing anti-maltose binding protein and anti-30 kDa lysyl oxidase antibodies allow assessment of lysyl oxidase processing activity. Thus, the intact 86 kDa fusion protein is recognized both by maltose binding protein antibodies, and antibodies raised against mature 30 kDa lysyl oxidase, whereas after procollagen C-proteinase processing the released 30 kDa lysyl oxidase is recognized only by lysyl oxidase antibodies. The 56 kDa maltose binding protein/lysyl oxidase propeptide product is recognized by maltose binding protein antibodies (Figure 14).

Fusion protein processing by purified recombinant BMP-1, mTLD, mTLL-1, and mTLL-2 was assayed in order to assess for efficiency of conversion by these different enzymes. Thirty ng of each purified enzyme was incubated with 7 μg of fusion protein for 45 min or for 4 hours, and products were subjected to PAGE and Western blotting with maltose binding protein and lysyl oxidase polyclonal antibodies as described in "Materials and Methods". As expected, incubations containing fusion protein and no added proteinase exhibited a single major 86 kDa band and sometimes an additional minor 84 kDa band when probed with either maltose binding protein (lane 1 in Figure 15, A) or lysyl oxidase (lane 1 in Figure 15, B and Figure 16) antibodies. Note that, as previously described (Feres-Filho et al. 1995, and Kagan 1986), use of the lysyl oxidase antibody results in artifactual bands at ~68 kDa, found in sample buffer alone and present in all lanes, despite affinity purification of the antibody (Figure 15 and Figure 16).

Incubation of the fusion protein with each of the enzymes resulted in processing of the 86 kDa protein. This is evident in Fig. 15, where decreased intensity of the 86 kDa fusion protein is accompanied by increased intensity of the 56 kDa maltose binding protein/lysyl oxidase pro-peptide fusion product in all reactions containing recombinant enzymes. The conversions are time-dependent, as greater conversion occurred after four hours incubation (compare Figure 15, and Figure 16). Production of 30 kDa lysyl oxidase is also time-dependent. Thus, 30 kDa lysyl oxidase was clearly detected in 45 minute incubations only in BMP-1 reactions (Figure 15), suggesting that BMP-1 more rapidly processes lysyl oxidase than mTLD, mTLL-1 or mTLL-2. By contrast, 30 kDa lysyl oxidase was found in reactions incubated for four hours containing BMP-1, mTLL-1, or mTLD (Fig. 16, lanes 3, 5, and 2, respectively).A small amount of 30 kDa lysyl oxidase was found in 4 hour incubations containing mTLL-2 (Fig. 16, lane 4).

DISCUSSION

Part One

The mechanisms of increased extracellular matrix production and accumulation in different forms of gingival overgrowth are only partially understood. It has recently been recognized that gingival overgrowth may partially depend on altered levels and activities of cytokines that normally help to maintain the balance between extracellular matrix production and resorption. Indeed, as noted, increased levels of bFGF, TGF-β, IL-6, PDGF, and IL-1, have been found in gingival overgrowth tissues caused by different drugs (Dill *et al.,* 1993; Iaccopino *et al.,* 1997; Plemmons *et al.,* 1996; Saito *et al.,*1997; Williamson *et al.,* 1994).

Evidence for increased collagen and non-collagen protein synthesis and accumulation in different forms of gingival overgrowth has been reported (Hassell and Hefti, 1991; Mariani *et al.*, 1996; Tipton *et al.*, 1997). Mature collagens and elastin contain lysine-derived cross-links necessary for normal function of the extracellular matrix. Cross-link formation depends on the extracellular enzyme lysyl oxidase. This enzyme catalyzes the oxidative deamination of lysine and hydroxylysine residues in collagen, and lysine residues in elastin, to generate reactive peptidyl aldehydes that undergo condensation reactions to ultimately form lysine-derived cross-links (Kagan, 1986; Kagan and Trackman, 1991). Increased cross-linking, lysyl oxidase activity, and lysyl oxidase protein are associated with fibrotic tissues in animal models and in human diseases, and studies support that increased levels of lysyl oxidase derived cross-links confer resistance to proteolysis and promote accumulation of collagen and elastin in the extracellular matrix (Kagan, 1986).

TGF-β1 promotes accumulation of extracellular matrix and insoluble collagen by different complementary mechanisms. For example, collagen synthesis and insoluble collagen accumulation are increased, and concomitantly, matrix-degrading proteases are inhibited by mechanisms that include altered production of plasminogen activators and of plasminogen activator inhibitors as well as matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (Trojanowska *et al.*, 1998). Proteoglycan and fibronectin synthesis are stimulated, and elastin biosynthesis is increased by TGF-β (Liu and Davidson, 1988). Consistent with these effects, our laboratory and others have shown that TGF-β1 increases lysyl oxidase enzyme activity and mRNA levels in different animal cell culture models (Boak *et al.*, 1994; Feres-Filho *et al.*, 1995; Shanley *et al.*, 1997). Results of our past study in gingival fibroblasts were surprising in that the time-course of stimulation of lysyl oxidase and collagen mRNA levels and lysyl oxidase activity levels were delayed (Hong *et al.*1999) compared to our previous studies performed in murine osteoblastic cells cultured under similar conditions (Feres-Filho *et al.*, 1995). We therefore considered the possibility that an intermediate factor induced by TGF-β1 might more directly stimulate collagen and/or lysyl oxidase production in human gingival fibroblasts.

Connective tissue growth factor is induced by TGF-β1, and has been found at elevated levels in fibrotic tissues and fibrous stroma surrounding mammary tumors (Frazier and Grotendorst, 1997; Igarashi *et al.*, 1996; Igarashi *et al.*, 1995; Ito *et al.*, 1998; Lasky *et al.*, 1998). CTGF is a member of a multigene family known as the CCN family characterized by conservation of 38 cysteine residues and a high degree of sequence homology among different family members (Boak, 1993). CTGF itself contains multiple structural motifs including an IGF binding domain, a von Willebrand factor repeat, and a thrombospondin repeat (Oemar and

Luscher, 1997). CTGF may have multiple biological activities, has been shown to stimulate extracellular matrix accumulation in NRK cells (Frazier *et al.*, 1996). Proteolytic fragments of CTGF found in uterine fluid are mitogenic for different cell types, and CTGF and homologues may contribute to chondrogenesis (Brigstock *et al.*, 1997; Nakanishi *et al.*, 1997; Nishida *et al.*, 1998; Wong *et al.*, 1997). Recent studies show that CTGF is a potent angiogenic factor (Babic *et al.*, 1999; Shimo *et al.*, 1998). CTGF expression and activity in gingival cells and tissues has not been previously studied.

We evaluated the hypothesis that CTGF may be induced by TGF-β1 in gingival cells and tissues, and that CTGF itself might stimulate insoluble collagen accumulation. In addition, the possibility that CTGF is elevated in hyperplastic gingival tissue was evaluated. Interestingly, we had found that CTGF mRNA levels are strongly induced in human gingival fibroblasts by TGF-β1, but not by IL-6 or bFGF (Hong *et al.,* 1999). In our present study, Western blotting of cell layers revealed elevated levels of 38 – 42 kDa CTGF in TGF-β1-treated cells, whereas media samples revealed in addition elevated levels of 20 kDa forms of CTGF. These results suggest that extracellular proteolytic processing of CTGF by human gingival fibroblastic cultures occurs. It seems possible that cell type or tissue-specific proteolytic processing of CTGF may result in fragments with differing biological activities that may partially account for the varied biological activities attributed to CTGF noted above. Treatment of human gingival fibroblasts with intact CTGF resulted in modest and slow stimulation of lysyl oxidase activity, and an increase in insoluble collagen accumulation by cultured human gingival fibroblasts. Exogenous administration of CTGF might result in a non-physiologic distribution of proteolytic fragments derived from CTGF, which may underestimate the true activity and role of endogenously produced CTGF in stimulating extracellular matrix production. In addition, synergistic action of

TGF-β1 and CTGF, and CTGF fragments may collectively account for regulation of lysyl oxidase and collagen observed for TGF-β1 administration alone. Cooperative interactions in the stimulation of collagen gene expression in smooth muscle cells by TGF-β1 and PDGF-A have been suggested (Halloran *et al.*, 1996). It is clear, however, that simple mediation of TGF-β1 effects by intact CTGF cannot fully account for the relatively slow induction of lysyl oxidase and collagen by TGF-β1. Future studies examining structure-function activities of CTGF and analysis for possible synergistic effects with TGF-β will be highly informative.

TGF-β1 (400 pM) increased collagen mRNA levels 3–4 fold in the presence and absence of CTGF blocking antibody. As noted, this antibody blocks the CTGF mitogenic activity in a human cell line. The lack of effect of this antibody in preventing or inhibiting TGF-β1 stimulated collagen mRNA levels supports the notion that following stimulation of CTGF levels, TGF-β1stimulates extracellular matrix accumulation by pathways independent of CTGF. The finding that CTGF itself stimulates lysyl oxidase enzyme activity and insoluble collagen accumulation indicates that CTGF does contribute to extracellular matrix accumulation. These findings suggest that TGF-β1 and CTGF have mostly independent roles in stimulating extracellular matrix production in human gingival fibroblasts. It is important to note that CTGF has additional known biological activities including mitogenic activity and angiogenic activity. Future studies will focus on whether these other activities of CTGF may be important in the biological functions and role of gingival CTGF.

It is interesting that CTGF stimulates lysyl oxidase enzyme activity without increasing lysyl oxidase mRNA levels. This finding predicts that CTGF in some way increases the amount of active lysyl oxidase by influencing either lysyl oxidase stability, or by stimulating secretion or processing of the inactive lysyl oxidase pro-enzyme. It is notable that pro-lysyl oxidase is

processed extracellularly principally by procollagen C-proteinase (Panchenko *et al.*, 1996), and that extracellular processing of lysyl oxidase contributes to the regulation of the level of active enzyme in some cell types (Feres-Filho *et al.*, 1995).

Human gingival overgrowth tissues contain detectable levels of CTGF protein. Differences in the cellular staining pattern between CTGF and TGF-β1 observed suggest that a simple *in vivo* stimulation of CTGF production exclusively by TGF-β1 does not account for the presence of CTGF. It should be pointed out that there is currently no information available regarding the relative stability of TGF-β1 and CTGF proteins *in vivo*. Thus, CTGF may not stably accumulate in all portions of tissues due to high turnover of CTGF. *In vitro* studies support the idea that CTGF may be rapidly turned over, as CTGF protein levels in TGF-β1-treated cells increased less dramatically than CTGF mRNA levels. Studies of CTGF protein stability in bovine endothelial cells indicate low CTGF stability in micro vessel endothelial cells, and higher stability in large vessel endothelial cells (Boes *et al.*, 1999). This further supports the notion that regulation of CTGF protein turnover contributes to the levels of CTGF accumulation both in vivo and in vitro. Alternatively, CTGF may be induced by TGF-β in a subset of gingival cells, whereas TGF-β is produced by a greater variety of cell types. This could partially account for the different patterns of cellular expression of TGF-β1 and CTGF in gingival sections.

In the *in vivo* part of our studies, we have investigated the abundance and the localization of CTGF in various drug-induced gingival overgrowth tissues and compared its intra- and extracellular distribution at different tissue areas. Our results demonstrate that drug-induced gingival overgrowth due to phenytoin therapy has significantly higher amounts of CTGF in all tissue areas compared to control and other drug-induced gingival overgrowth samples. This is the first time to evaluate the possible role of CTGF in different drug-induced gingival

overgrowth samples and to compare CTGF levels in various tissue areas on the histological sections. The abundance of CTGF in the extracellular area was in parallel with the findings at the intracellular level. Phenytoin samples had significantly higher staining for CTGF in both the cells and the extracellular matrix suggestive of a double function of this growth factor in inducing tissue changes. Phenytoin samples showed components that are more fibrotic compared to the other overgrown tissues while less inflammatory cell counts were detected. This indicates that CTGF expression in phenytoin induced drug-induced gingival overgrowth representing mainly a fibrotic overgrowth, underlies an important pathological change that is related to the action of fibroblasts. Indeed, it has been suggested that CTGF could stimulate fibroblast proliferation and insoluble collagen accumulation (Hong et al. 1999). These suggestions are supported to a certain extent by the data from the present study by simultaneous finding of an increase of fibrosis and CTGF expression. This aspect, however, should be verified by further *in vitro* and *in vivo* studies.

Dexamethasone, a potent glucocorticoid, stimulates the CTGF expression in normal tissues and organs but not in inflamed areas (Dammeier et al. 1998). Based on this finding, it may be predicted that in cyclosporin A-induced gingival overgrowth, where patients are also under simultaneous glucocorticoid treatment, CTGF expression would be induced. Our findings indicate that the abundance of CTGF in cyclosporin A samples is not statistically different from the control tissues. This suggests that the inflammatory component of the gingival overgrowth in these samples should be considered as an important reflection of the clinical lesion. Since CTGF levels went down at the inflammatory stage of scleroderma, it could be further thought that the inflammation might suppress the stimulation of CTGF. Observation of an increased inflammatory cell counts in cyclosporin A tissue samples also supports this suggestion.

Previously, it was reported that although 60% of the gingival overgrowth correlates with the fibrosis in cyclosporin A -treated patients, 40% of the lesion is related to the inflammation suggesting the local effect of the bacterial impact (Kantarci 1999).

Investigations on the relationship between CTGF and TGF-β have showed that TGF-β specifically stimulates CTGF in fibroblasts (Igarashi *et al.,* 1995; Grotendorst 1996). While TGF-β acts as an initiator of the repair process, CTGF functions as an autocrine and paracrine signaling molecule to maintain and perhaps amplify and synchronize the response of fibroblastic cells in the tissue (Frazier, 1996). Similar to TGF-β1, CTGF is able to stimulate type I collagen, integrin α5, and fibronectin in fibroblasts. Thus, these two proteins would function in a regulatory cascade in healing or regenerating tissue (Igarashi, 1993; Frazier, 1996) suggesting their importance in the formation of fibrotic tissues. This assumption has been further confirmed in skin sclerosis where the existence of CTGF gene expression is strongly correlated with the lesion (Igarashi, 1996). While CTGF has been continuously detected in these lesions, TGF-β regulation has been seen only in the very earliest stage. In addition, the subcutaneous injection of TGF-β and CTGF into the mice caused granulation tissue and fibrosis (Frazier, 1996). It has been proposed that in addition to transcriptional regulation of CTGF gene expression by TGF-β in some cell types, there are one or more control points in the CTGF biosynthetic pathway, that serve to regulate qualitative and quantitative aspects of CTGF secretion (Steffen, 1998). Although it has initially been suggested that CTGF is associated with the presence of TGF-β, it is now accepted this is a cascade-like relationship rather than a co-expression pattern. This assumption has also been justified by our findings. We have failed to identify the simultaneous abundance of these two growth factors and there was no correlation between them. Moreover, the differences in TGF-β1 expression between the groups were not statistically significant in

most tissue areas. This finding, however, does not exclude the possibility of a cascade-like relationship between CTGF and TGF-β1 and demands the conduct of longitudinal, well-controlled experimental studies.

In summary, our findings show that CTGF is strongly stimulated by TGF-β1 in human gingival fibroblasts. This is perhaps very important, proving that CTGF is a functional cytokine released by human gingival fibroblasts. Indeed, the pattern of CTGF stimulation by TGF-β1, as it is stated in the literature review section, is very consistent with findings of other investigators working on other systems and fibroblastic cell lines. Our results indicated that CTGF is initially elevated at four hours at RNA level, then it is maximally elevated at 24 hours and stayed elevated at 48 hours. We also detected 38-42 kDa CTGF at protein level consistent to the RNA expression patterns. Moreover, the presence of a 20 kDa CTGF fragment in human gingival fibroblasts has great importance shoving one more time that CTGF is highly functional and due to that undergoes proteolytic cleavage. This kind of processing perhaps happens after certain functional activities, such as possible stimulation of gingival fibroblasts for collagen synthesis. Perhaps CTGF functions on ECM as a mediator of TGF-β in human gingival fibroblasts. As an evidence of CTGF functions, we also showed the stimulation of long-term collagen accumulation by exogenous CTGF addition.

One of our important findings, *in vivo,* is the distinction between phenytoin-induced gingival overgrowth and nifedipine and cyclosporin A-induced gingival overgrowth. We can certainly say that phenytoin-induced gingival overgrowth is much more fibrotic in nature and local inflammation and oral hygiene of the patients has much less importance. We also found that in phenytoin-induced gingival overgrowth samples exhibited inflammatory cell counts similar to the control group. Consequently, patients suffering of phenytoin-induced gingival

overgrowth should undergo gingivectomy surgery rather than long term recall type scaling and root planning treatment. Nifedipine-induced gingival overgrowth also exhibits, clinical and microscopic characteristics of fibrosis, even though inflammation and poor oral hygiene are triggering factors for nifedipine-induced gingival overgrowth. In this case, patients should practice strict oral hygiene supplemented by professional scaling and root planning before any need of surgical therapy is determined. Opposite to the phenytoin- induced gingival overgrowth, cyclosporin A-induced gingival overgrowth tissues are more inflamed than fibrotic, since intracellular CTGF numbers are much less and inflammatory cell counts are much more then phenytoin group and control tissues. In this case, the clinical approach should be more conservative. Oral hygiene should be very much emphasized and scaling and root planning should be initially performed every month and that longer intervals in the course of treatment. The systemic status of cyclosporin A-induced gingival overgrowth patients should also be considered in clinical applications. In that context, the choice of clinical treatment modality has great importance, since bacteremia may be caused by vigorous treatments and eventually patient's health may be jeopardized. Our findings suggest that CTGF may be a crucial cytokine that stimulates fibrotic gingival overgrowth, and CTGF may be part of a cascade of molecular events that lead to drug-induced gingival overgrowth. The presence or the absence of CTGF may also determine what kind of clinical treatment modality should be chosen.

Future studies may address, time and dose dependent expression of TGF-β's and CTGF in gingiva in developing phenytoin, nifedipine, or cyclosporin-induced gingival hyperplasia in experimental animals models. Experimental animal models may also help determining whether TGF-β1 strongly induces CTGF in the oral environment. In addition, detailed clinical characterization coupled to histological and immunohistochemical analyses of human gingival

overgrowth tissues may result in new identification and classification of differences among these tissues that may provide insight into the etiology and treatment of different forms of gingival overgrowth. An *in vitro* and *in vivo* blockage of CTGF may also explain more what its functions are.

One of the well known intracellular messenger systems is the adenylate cyclase system. In this system, chemical signals like neurotransmitters and hormones are recognized by several different kinds of membrane receptors triggering cell membrane bound enzyme adenylate cyclase. Then, adenylate cyclase converts intracellular ATP to cAMP eventually causing intracellular events (Champe *et al.1994*). As it is stated before, the presence of a TGF-β response element in the human CTGF promoter was shown. The TGF-β response element shares partial sequence homology with cAMP response element. TGF-β activation of CTGF through the TGF-β response element was inhibited by cAMP analogues and agents that elevate intracellular cAMP. Furthermore, increased intracellular cAMP inhibited TGF-β stimulation of collagen synthesis. The stimulation of CTGF by TGF-β was also blocked *in vivo* by cAMP-elevating agents. Indeed, cAMP-elevating agents suppressed CTGF stimulation by TGF-β and collagen synthesis in NRK cells. The addition of CTGF did not stimulate collagen synthesis in cells treated with both TGF-β and cAMP-elevating agents. Interestingly, this blockage did not affect TGF-β stimulated changes in the morphology in NRK and NIH/3T3 fibroblasts, suggesting that other TGF-β signaling pathways such as Smads are still active. Additionally, possible CTGF/ TGF-β dependent and independent pathways are described. (Kothapalli et al. 1998, Duncan et al 1999). It appears that cAMP-elevating agents may be used as therapeutic agents, since they specifically block TGF-β stimulation of CTGF for collagen synthesis. In addition, possible CTGF/ TGF-β dependent and independent pathways are described. This may suggest that CTGF/

TGF-β pathway may only be stimulated when there is a fibrotic event while independent pathways are also effective for regular cellular functions. Furthermore, the other functions of TGF-β are not blocked by cAMP-elevating agents increasing their therapeutic value. Indeed, this seems to be more important for phenytoin induced gingival overgrowth since the biopsies from patients taking phenytoin exhibited much abundant fibrotic component. Furthermore, it appears that altering CTGF stimulation by TGF-β with cAMP-elevating agents may not effect other cellular functions. Further investigation is needed in order to determine, whether human gingival fibroblasts follow a similar pathway, or whether a different, unknown mechanism is active. Then, since our ultimate goal is to solve and treat biological questions, an *in vivo* model should be established and detailed molecular analysis should be made. This may very well ease the understanding of the changes as a cascade of events in drug-induced gingival overgrowth, both at microscopic and molecular level.

It is also interesting to show whether other CCN early gene family members such as cyr61, is also present in drug-induced gingival overgrowth samples. The pattern of cyr61 presence may also occur as a function of different drug therapies that stimulate gingival overgrowth. Even though cyr61 has homology and similar conserved sequences to CTGF, it has a serum response element in the promoter sequence rather than a TGF-β response element. It is known that TNF-α stimulates cyr61. Elevated levels of inflammatory mediators such as TNF-α may activate transcription of cyr61 by binding to the serum response element. This may especially be the case for cyclosporin A-induced gingival overgrowth since it exhibits high levels of inflammatory cells. This may also explain why CTGF levels are much lower then the other two gingival overgrowth groups.

It is been shown that mechanical stimulation induces CTGF expression in rat osteocytes (Yamashiro et al. 2001). In this context, exogenous addition of CTGF in to critical size mouse, and rat calvaria; or rabbit tibia and may enhance the understanding of CTGF functions in ECM stimulation, and/or osteogenesis. Further clinical applications, could be the tried by using rCTGF in combination with bone allografts during guided bone regeneration procedures.

DISCUSSION

Part Two

Two recent discoveries lead to the notion that certain enzymes, which provide procollagen C-proteinase enzymes, may be key in initiating and controlling a cascade of extracellular transformations critical for extracellular matrix deposition. The two discoveries are (a) that enzymes with procollagen C-proteinase activity have multiple extracellular substrates, and (b) that the *Bmp1* gene, related to genes that regulate morphogenetic patterning in non-mammalian species, encodes procollagen C-proteinases. In addition to the biosynthetic processing of procollagen types I, II, and III, procollagen C-proteinase enzymes proteolytically process pro-lysyl oxidase (Panchenko *et al.,* 1996), inactivate Chordin to release growth factors from latent complexes (Scott *et al.,* 1999; Mullins, 1998), and may also process pro-α1(V) collagen (Imamura *et al,* 1998) and laminin-5 (Amano *et al.,* 2000). The concept of a key enzymatic activity that controls a series of subsequent activities leading to a biological phenotype is well know in catabolic cascades, in which extracellular matrix destruction is initiated by collagenases (MMP-1, MMP-8, MMP-13), or plasmin (Nagase and Woessner, 1999). The existence of anabolic or synthetic extracellular enzymatic cascades now seems likely following characterization of developmentally regulated enzymes such as those with procollagen C-proteinase activity.

As with key catabolic enzymes such as the collagenases, a certain degree of redundancy appears to exist for anabolic enzymes, where key enzyme activities may be encoded by different genes in a tissue-specific and developmentally regulated manner. The present study investigates the ability of proteinases derived from the *Bmp1* gene itself, and of two highly similar proteinases encoded by *Bmp1*-related mammalian genes to process pro-lysyl oxidase *in vitro*. In addition, the effects of the absence of proteases encoded by the *Bmp1* gene on lysyl oxidase activity and biosynthesis was studied in fibroblasts cultured from wild type and *Bmp1*-null mice. The *in vitro* data indicate that BMP-1, mTLD, mTLL-1 and mTLL-2 enzymes process pro-lysyl oxidase with different efficiencies and, perhaps, in somewhat different ways. Thus, BMP-1 is the

most efficient at processing pro-lysyl oxidase to produce the mature enzyme, whereas mTLL-2 does less productively process pro-lysyl oxidase into a stable active form. The observed trend in relative pro-lysyl oxidase processing parallels the previously reported activities of these enzymes against type I and type II procollagens (Prockop *et al.,* 1998). Thus, the high efficiency of BMP-1 processing of both procollagens and pro-lysyl oxidase points towards BMP-1 as a principal activity in the biosynthesis of functional insoluble collagen. Previously reported expression patterns of BMP-1, mTLD, mTLL-1, and mTLL-2 in developing hindlimbs indicate BMP-1 and mTLD to be expressed at high levels throughout limb mesenchyme, while mTLL-1 expression appeared more restricted to perichondrium or periosteum, and mTLL-2 expression appeared principally in skeletal muscle. Thus, patterns of expression of BMP-1 and mTLD in highly collagenous tissues are also consistent with a principal role in lysyl oxidase and mature collagen biosynthesis. The more restricted expression patterns of mTLL-1 and mTLL-2 may indicate secondary or more specialized roles for these products in the normal development of collagenous extracellular matrices.

The analysis of N-terminal sequence of lysyl oxidase fusion protein after the processing by BMP-1 , mTolloid, mTLL-1, mTLL-2 produced 30 kDa lysyl oxidase. Furthermore, the availability of embryonic fibroblasts, and tissue specimens from *Bmp1* , *mTLL-1* and *mTLL-2* null mice defined more clearly which gene product is essential for lysyl oxidase processing (Uzel et. al 2001).

In summary, our findings support the idea BMP-1 having versatile functions. This fits this enzyme in a much larger biological picture, such previous findings as BMP-1 being the inhibitor of chordin, the inhibitor of BMP-4, supports the ubiquitous presence of BMP-1. Moreover, mTLL-1 and mTLL-2 are partially able to replace BMP-1. This is also evidence showing that when BMP-1 is lacked during its other functions, other molecules such as MLL-1

can easily replace it. It is also proves the likeliness of BMP-1 versatility in other functions, other than pro-collagen, pro-lysyl oxidase cleavage and chordin inhibition. In that context, even though BMP-1 is not a TGF-β superfamily member, BMP-1 may also have many synergistic functions with the other BMP's.

In our studies it is showed for the first time, that pro-lysyl oxidase is the first known substrate for mTLL-2. Further studies on mTTL-2 should be also done, experimentation of mTLL-2 null and mTLL-2 and BMP-1 null mouse cells and animals may illuminate more its functions.

REFERENCES

Aiba, T; Akeno, N; Kawane, T; Okamoto, H; Horiuchi, N; (1996) Matrix Metalloproteinases-1,and -8 and TIMP-1 mRNA Levels In Normal And Diseased Human Gingivae; *Eur J Oral Sci* Oct-Dec;104(5-6):562-9

Almassian, B; Trackman, P. C.; Iguchi, H.; Boak, A.; Calvaresi, D.; and Kagan, H. M. ; (1991) Induction of lung lysyl oxidase activity and lysyl oxidase protein by exposure of rats to cadmium chloride: properties of the induced enzyme.; *Conn. Tiss. Res.* 25, 197- 208

Amano, S.; Takahara, K.; Gerecke, D.; Nishiyama, T.; Lee, S.; Greenspan, D.S.; and Burgeson, R.E. (1996) *Mol. Biol. Cell* 7, Suppl., 58a (Abstr. 338)

Babic, A.; M. Chen.; C.-C.; and Lau, L. F. (1999). Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin avb3, promotes endothelial cell survival and induces angiogenesis in vivo. *Mol. Cell. Biol.* 19, 2858 - 2966.

Bedell-Hogan, D.; Trackman, P.; Abrams, W.; Rosenbloom, J.; and Kagan, H. (1993). Oxidation, cross-linking, and insolubilization of recombinant tropoelastin by purified lysyl oxidase. *J. Biol. Chem.* 268, 10345 - 10350.

Blader, P.; Rastegar, S.; Fischer, N.; Strahle, U. (1997) Cleavage of the BMP-4 antagonist chordin by zebrafish tolloid. *Science* Dec 12;278(5345):1937-40

Boak, A.; Roy, R.; Berk, J.; Taylor, L.; Polgar, P.; Goldstein, R. H.; and Kagan, H. M. (1994). Regulation of lysyl oxidase expression in lung fibroblasts by TGF-b1 and prostaglandin E2. *Am. J. Respir. Cell Mol. Biol.* 11, 751 - 755.

Boes, M.; Dake, B. L.; Booth, B. A.; Erondu, N. E.; Oh, Y., Hwa, V.; Rosenfeld, R.; and Bar, R. S. (1999). Connective tissue growth factor (IGFBP-rP2) expression and regulation in cultured bovine endothelial cells. *Endocrinology* 140, 1575-80.

Bork, P. (1993). The molecular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett.* 327, 125 - 130.

Boyd, C. D.; Christiano, A. M.; Pierce, R. A.; Stolle, C. A.; and Deak, S. B. (1991). Mammalian tropoelastin: multiple domains of the protein define an evolutionarily divergent amino acid sequence. *Matrix* 11, 235 - 241.

Brigstock, D. R.; Steffen, C. L.; Kim, G. Y.; Vegunta, R. K.; Diehl, J. R.; and Harding, P. A. (1997). Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. Identification as heparin- regulated Mr 10,000 forms of connective tissue growth factor. *J Biol Chem* 272, 20275-82.

Carranza, F.A., Newman, M.G.(1996)W.B. Saunders Company, Clinical Periodontology,8th Edition

Casetta, I.; Granieri, E.; Desidera M.; Monetti V.C.; Tola M.R.; Paolino E.; Govani V.; Calura G. (1997). Phenytoin-induced gingival overgrowth: a community-based cross-sectional study in Ferrara,Italy. *Neuroepidemiology;*16:296-303

Centrella, M.; Casinghino, S.; Ignotz, R.; and McCarthy, T. L. (1992). Multiple regulatory effects by transforming growth factor-β on type I collagen from fetal rat bone. *Endocrinology* 131, 2863 - 2872.

Champe P.C.; Harvey R.A.(1994) Biochemistry2nd Edition ,Lippincott's Illustrated Reviews, Lippincott-Raven Publishers

Childs, S.R.; O'Connor M.B. (1994). Two domains of the tolloid protein contribute to its unusual genetic interaction with decapentaplegic. *Dev Biol Mar*;162(1):209-20

Chanoki, M.; Ishii, M.; Kobayashi, H.; Fushida, H.; Yashiro, N.; Hamada, T.; and Ooshima, A.(1995). Increased expression of lysyl oxidase in skin with scleroderma. *Brit. J. Dermatol.* 133, 710 - 715.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15, 532 - 536.

Clark, T.G.; Conway, S.J.; Scott, I.C.; Labosky, P.A.; Winnier, G.; Bundy, J.; Hogan, B.L.; Greenspan, D.S.(1999). Mammalian Tolloid-like 1 gene, Tll1, is necessary for normal septation and positioning of the heart. *Development* Jun;126(12):2631-42

Connors, S.A.; Trout, J.; Ekker, M.; Mullins, M.C.(1999). The role of tolloid/mini fin in dorsoventral pattern formation of the zebrafish embryo. *Development* Jun;126(14):3119-30

Cronshaw, A. D.; Fothergill-Gilmore, L. A.; and Hulmes, D. J. S. (1995) The proteolytic processing site of the precursor of lysyl oxidase. *Biochem. J* 306; 279 – 84

Dahlof, G.; Modeer, T.; Reinholt, F.P.; Wikstrom,B.; Hjerpe, A.(1986). Proteoglycans and glycosaminoglycans in phenytoin-induced gingival overgrowth. *J. of Perio Res* 21:13-21

Dammeier, J.; Brauchle, M.; Falk, W.; Grotendorst, G. R.; and Werner, S. (1998). Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *Int J Biochem Cell Biol* 30, 909-22.

de Waal, J.; Olivier, A.; van Wyk, C. W.; and Maritz, J. S. (1997). The fibroblast population in oral submucous fibrosis. *J Oral Pathol Med* 26, 69-74.

Dill, R. E.; Miller, E. K.; Weil, T.; Lesley, S.; Farmer, G. R.; and Iacopino, A. M. (1993). Phenytoin increases gene expression for platelet-derived growth factor B chain in macrophages and monocytes. *J Periodontol* 64, 169-73.

Dill,R.E.;and Iacopino, A. M. (1997). Phenytoin induced myofibroblasts *JPeriodontol*68,375-80

Duncan MR, Frazier KS, Abramson S, Williams S, Klapper H, Huang X, Grotendorst GR.Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP(1999). *FASEB J* Oct;13(13):1774-86

Ellis, J.S.; Seymour, R.A.; Steele, J.G.; Robertson, P.; Butler, T.J.; Thomason, J.M.(1999). Prevalence of gingival overgrowth induced by calcium channel blockers: a community-based study. *J Periodontol* Jan;70(1):63-7

Feres-Filho, E. J.; Choi, Y. J.; Han, X.; Takala, T. E. S.; and Trackman, P. C. (1995). Pre- and post-translational regulation of lysyl oxidase by transforming growth factor b1 in osteoblastic MC3T3-E1 cells. *J. Biol. Chem.* 270, 30797 - 30803.

Ferguson, E.L.; Anderson, K.V.(1992). Localized enhancement and repression of the activity of the TGF-beta family member, decapentaplegic, is necessary for dorsal-ventral pattern formation in the Drosophila embryo. *Development* Mar;114(3):583-97

Franceschi, R. T.; Iyer, B. S.; and Cui, Y. (1994). Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in MC3T3-E1 cells. *J. Bone Min. Res.* 9, 843 - 854.

Frazier, K.; Williams, S.; Kothapalli, D.; Klapper, H.; and Grotendorst, G. R. (1996). Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 107, 404-11.

Frazier, K. S.; and Grotendorst, G. R. (1997). Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. *Int J Biochem Cell Biol* 29, 153-61.

Fukagawa, M.; Suzuki, N.; Hogan, B.L.; Jones, C.M. (1994) Embryonic expression of mouse bone morphogenetic protein-1 (BMP-1), which is related to the Drosophila dorsoventral gene tolloid and encodes a putative astacin metalloendopeptidase. *Dev Biol* May;163(1):175-83

Gabbiani, G.(1994) Modulation of fibroblastic cytoskeletal features during wound healing and fibrosis. *Pathol Res Pract.* (1994) Oct;190(9-10):851-3.

Genovese, C.; Rowe, D.; and Kream, B. (1984). Construction of DNA sequences complementary to rat a1 and a2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D. *Biochemistry* 23, 6210 - 6216.

Gindre, D.; Peyrol, S.; Raccurt, M.; Sommer, P.; Loire, R.; Grimaud, J. A.; and Cordier, J. F. (1995). Fibrosing casculitis in Wegener's granulomatosis: ultrastructural and immunohistochemical analysis of the vascular lesions. *Virchows Arch.* 427, 385 - 393.

Halloran, B. G.; Byung, J. S.; and Baxter, T. (1996). Platelet-derived growth factor is a cofactor in the induction of a-1(I) procollagen expression by transforming growth factor-b1 in smooth muscle cells. *J. Vasc. Surg.* 23, 767 - 774.

Hassell, T.M.; Page, R.C.; Lindhe J. (1978) Histologic evidence for impaired growth control in diphenylhydantoin gingival overgrowth in man. *Arch Oral Biol*;23(5):381-4

Hassell, T.; and Hefti, A. F. (1991). Drug-induced gingival overgrowth: Old problem, new problem. *Crit. Rev. Oral Biol. Med.* 2, 103 - 137.

Hassell, T.(1982)Evidence foer production of an Inactive Collagenase by Fibroblasts from Phenytoin-Enlarged Human gingivae *J of Oral Pathol.* 11,310-317

Hassell, T; and Gilbert, G.H. (1983). Phenytoin Sensitivity of Fibroblasts as the Basis for Susceptibility to Gingival Enlargement *An J Pathol* ,112:218-223

Hillis, D.; and Dixon, T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quaterly Review of Biology* 66, 411 - 453.

Hong, H.H.; Uzel, M.I.; Duan, C.; Sheff, M.C.; Trackman, P.C. (1999). Regulation of lysyl oxidase, collagen, and connective tissue growth factor by TGF-beta1 and detection in human gingiva. *Lab Invest.* Dec;79(12):1655-67.

Hulmes, D.J.; Mould, A.P.; and Kessler, E. (1997). The CUB Domains Of Procollagen C-Proteinase Enhancer Control Collagen Assembly Solely By Their Effect On Procollagen C-Proteinase/Bone Morphogenetic Protein-1.*Matrix Biol* 16(1), 41-5

Iacopino, A. M.; Doxey, D.; Cutler, C. W.; Nares, S.; Stoever, K.; Fojt, J.; Gonzales, A.; and Dill, R. E. (1997). Phenytoin and cyclosporine A specifically regulate macrophage phenotype and expression of platelet-derived growth factor and interleukin-1 in vitro and in vivo: possible molecular mechanism of drug-induced gingival hyperplasia. *J Periodontol* 68, 73-83.

Igarashi, A.; Nashiro, K.; Kikuchi, K., Sato, S.; Ihn, H.; Fujimoto, M.; Grotendorst, G. R.; and Takehara, K. (1996). Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J Invest Dermatol* 106, 729-33.

Igarashi, A.; Nashiro, K.; Kikuchi, K.; Sato, S.; Ihn, H.; Grotendorst, G. R.; and Takehara, K. (1995). Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol* 105, 280-4.

Igarashi, A.; Okochi, H.; Bradham, D. M.; and Grotendorst, G. R. (1993). Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell* 4, 637-45.

Ingman, T.; Sorsa, T.; Michaelis, J.; Konttinen, Y.T. (1994). Immunohistochemical study of neutrophil- and fibroblast-type collagenases and stromelysin-1 in adult periodontitis. *Scand J Dent Res* Dec;102(6):342-9

Imamura, Y.; Steiglitz, B. M.; and Greenspan, D. S. (1998) Bone morphogenetic protein-1 processes the NH_2-terminal propeptide, and a furin-like pro-protein convertase processes the COOH-terminal propeptide of pro-alpha1(V) collagen. *J Biol Chem* 273(42), 27511-7

Ingman, T.; Tervahartiala, T.; Ding, Y.; Tschesche, H.; Haerian, A.; Kinane, D.F.; Konttinen, Y.T.; Sorsa, T.(1996) Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *J Clin Periodontol* Dec;23(12):1127-32

Ito, Y.; Aten, J.; Bende, R. J.; Oemar, B. S.; Rabelink, T. J.; Weening, J. J.; and Goldschmeding, R. (1998). Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int* 53, 853-61.

Jang, W.; Hua, A.; Spilson, S. V.; Miller, W.; Roe, B. A.; and Meisler, M. H. (1999) Comparative sequence of human and mouse BAC clones from the Mnd2 region of chromosome 2p13. *Genome Res* 9(1), 53-61

Janitz, M.; Heiser, V.; Bottcher, U.; Landt, O.; Lauster, R.(1998) Three alternatively spliced variants of the gene coding for the human bone morphogenetic protein-1 *J Mol Med* Feb;76(2):141-6

Jourdan-Le Saux, C.; Le Saux, O.; Donlon, T.; Boyd, C. D.; and Csiszar, K. (1998) The human lysyl oxidase-related gene (LOXL2) maps between markers D8S280 and D8S278 on chromosome 8p21.2-P21.3. *Genomics* 51(2), 305-7

Jourdan-Le Saux, C.; Tronecker, H.; Bogic, L.; Bryant-Greenwood, G. D.; Boyd, C. D.; and Csiszar, K. (1999) The LOXL2 Gene Encodes A New Lysyl Oxidase-Like Protein And Is Expressed At High Levels In Reproductive Tissues. *J Biol Chem* 274(18), 12939-44

Kessler, E.; Takahara, K.; Biniaminov, L.; Brusel, M.; and Greenspan, D. S. (1996) *Science* 271, 360 - 62

Kagan, H. (1986). Characterization and regulation of lysyl oxidase. *(R. P. Mecham, Ed.),*Biology and Regulation of Extracellular Matrix: A Series. Regulation of Matrix Accumulation Vol. 1,Orlando Academic Press,321 - 398.

Kagan, H. M.; and Trackman, P. C. (1991). Properties and function of lysyl oxidase. *Am. J. Resp. Cell Mol. Biol.* 5, 206 - 210.

Kenyon, K.; Modi, W. S.; Contente, S., and Friedman, R. M. (1993) A novel human cDNA with a predicted protein similar to lysyl oxidase maps to chromosome 15q24-Q25. *J Biol Chem* 268(25), 18435-7

Kubota, T.; Nomura, T.; Takahashi, T.; Hara, K.(1996) Expression of mRNA for matrix metalloproteinases and tissue inhibitors of metalloproteinases in periodontitis-affected human gingival tissue. : *Arch Oral Biol* Mar;41(3):253-62

Kumar, S.; Hand, A.T.; Connor, J.R.; Dodds, R.A.; Ryan, P.J.; Trill, J.J.; Fisher, S.M.; Nuttall, M.E.; Lipshutz, D.B.; Zou, C.; Hwang, S.M.; Votta, B.J.; James, I.E.; Rieman, D.J.; Gowen, M.; Lee, J.C.(1999) Identification and cloning of a connective tissue growth factor-like cDNA from human osteoblasts encoding a novel regulator of osteoblast functions.*J Biol Chem* Jun 11;274(24):17123-31

Lasky, J. A.; Ortiz, L. A.; Tonthat, B.; Hoyle, G. W.; Corti, M.; Athas, G.; Lungarella, G.; Brody, A.; and Friedman, M. (1998). Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis. *Am J Physiol* 275, L365-71.

Lee, S.;, Solow-Cordero, D. E.; Kessler, E.; Takahara, K., and Greenspan, D. S. (1997). Transforming growth factor-beta regulation of bone morphogenetic protein-1/procollagen C-proteinase and related proteins in fibrogenic cells and keratinocytes. *J Biol Chem* 272, 19059-66.

Lepage, T.; Ghiglione, C.; Gache, C.(1992) Spatial and temporal expression pattern during sea urchin embryogenesis of a gene coding for a protease homologous to the human 1protein BMP-1 and to the product of the Drosophila dorsal-ventral patterning gene tolloid: *Development* Jan;114(1):147-63.

Li, S. W.; Sieron, A. L.; Fertala, A.; Hojima, Y.; Arnold, W. V.; and Prockop, D. J. (1996). The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1.*Proc Natl Acad Sci U S A* 93(10), 5127-30

Liu, J.; and Davidson, J. M. (1988). The elastogenic effect of recombinant transforming growth factor beta on porcine aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* 154, 895 - 901.

Mariani, G.; Calastrini, C.; Carinci, F.; Bergamini, L.; Calastrini, F.; and Stabellini, G. (1996). Ultrastructural and histochemical features of the ground substance in cyclosporin A-induced gingival overgrowth. *J. Periodontol.* 67, 21 - 27.

Mariani, T. J.; Trackman, P. C.; Kagan, H. M.; Eddy, R. L.; Shows, T. B.; Boyd, C. D.; and Deak, S. B. (1992). Complete derived amino acid sequence of human lysyl oxidase and assignment of the gene to chromosome 5. *Matrix* 12, 242 - 248.

Marqués, G.; Musacchio, M.; Shimell, M.J.; Wünnenberg-Stapleton, K.; Cho, K.W.Y.; and O'Connor, M.B. (1997) Production of a DPP activity gradient in the early drosophila embryo through the opposing actions of the SOG and TLD proteins. *Cell* 91, 417-426

Meisler, N.; Keefer, K. A.; Ehrlich, H. P.; Yager, D. R.; Myers-Parrelli, J.; and Cutroneo, K. R. (1997). Dexamethasone abrogates the fibrogenic effect of transforming growth factor-beta in rat granuloma and granulation tissue fibroblasts. *J Invest Dermatol* 108, 285-9.

Miller, C. S. and. Damm, D.D. (1992) Incidence of Verapamil-Induced Gingival Hyperplasia in a Dental Population, *J of Perio* : 63:453-456.

Mullins, M. C. (1998) Holy tolloido: tolloid cleaves SOG/chordin to Free DPP/BMP's. *Trends Genet* 14(4), 127-9

Nagase, H.; and Woessner, J. F. Jr. (1999) Matrix Metalloproteinases *J Biol Chem* 274, 21491 - 4

Nguyen, T.; Jamal, J.; Shimell, M.J.; Arora, K.; O'Connor, M.B. (1994) Characterization of tolloid-related-1: a BMP-1-like product that is required during larval and pupal stages of Drosophila development.*Dev Biol* Dec;166(2):569-86

Nakanishi, T.; Kimura, Y.; Tamura, T.; Ichikawa, H.; Yamaai, Y.; Sugimoto, T.; and Takigawa, M. (1997). Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. *Biochem Biophys Res Commun* 234, 206-10.

Nakanishi, Y.; Ochiai, A.; Shimoda, T.; Yamaguchi, H.; Tachimori, Y.; Kato, H., Watanabe, H.; and Hirohashi, S. (1999). Heterotopic sebaceous glands in the esophagus: histopathological and immunohistochemical study of a resected esophagus. *Pathol Int* 49, 364-8.

Nishida, T.; Nakanishi, T.; Shimo, T.; Asano, M.; Hattori, T.; Tamatani, T.; Tezuka, K.; and Takigawa, M. (1998). Demonstration of receptors specific for connective tissue growth factor on a human chondrocytic cell line (HCS-2/8). *Biochem Biophys Res Commun* 247, 905-9.

Oemar, B. S.; and Luscher, T. F. (1997). Connective tissue growth factor. Friend or foe? *Arterioscler. Thromb. Vasc. Biol.* 17, 1483 - 1489.

Oemar, B. S.; Werner, A.; Garnier, J. M.; Do, D. D.; Godoy, N.; Nauck, M.; Marz, W.; Rupp, J.; Pech, M.; and Luscher, T. F. (1997). Human connective tissue growth factor is expressed in advanced atherosclerotic lesions. *Circulation* 95, 831-9.

Panchenko, M. V.; Stetler-Stevenson, W. G.; Trubetskoy, O. V.; Gacheru, S. N.; and Kagan, H. M. (1996). Metalloproteinase activity secreted by fibrogenic cells in the processing of pro-lysyl oxidase. *J. Biol. Chem.* 271, 7113 - 7119.

Penarrocha-Diago, M.; Bagan-Sebastian, V.; and Vera-Sempere, F.(1990) Diphenylhydantoin-induced gingival overgrowth in man: a clinico-pathological study *J Periodontol* Sep (571-574).

Peyrol, S.; Raccurt, M.; Gerard, F.; Gleyzal, C.; Grimaud, J. A.; and Sommer, P. (1997). Lysyl oxidase gene expression in the stromal reaction to in situ and invasive ductal breast carcinaoma. *Am. J. Pathol.* 150, 497 - 507.

Piche, J. E.; Carnes, D. L. Jr.; and Graves, D. T. (1989). Initial characterization of cells derived from human periodontia. *J Dent Res* 68, 761-7.

Plemons, J. M.; Dill, R. E.; Rees, T. D.; Dyer, B. J.; Ng, M. C.; and Iacopino, A. M. (1996). PDGF-B producing cells and PDGF-B gene expression in normal gingival and cyclosporine A-induced gingival overgrowth. *J Periodontol* 67, 264-70.

Prockop, D. J.; Sieron, A. L.; and Li, S. W. (1998) Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling *Matrix Biol* 16(7), 399-408

Raftery, L.A.; Twombly, V.; Wharton, K.; Gelbart, W.M.(1995) Genetic screens to identify elements of the decapentaplegic signaling pathway in Drosophila. *Genetics* Jan;139(1):241-54

Rucker, R. B.; Riggins, R. S.; Laughlin, R.; Chan, M. M.; Chen, M.; and Tom, K. (1975) *J. Nutrition* 105, 1062 – 70

Saito, K.; Mori, S.; Iwakura, M.; and Sakamoto, S. (1996). Immunohistochemical localization of transforming growth factor b, basic fibroblast growth factor and heparan sulfate glycosaminoglycan in gingival hyperplasia induced by nifedipine and phenytoin. *J. Periodont. Res.* 31, 545 - 555.

Saito, H.; Papaconstantinou, J.; Sato, H.; and Goldstein, S. (1997) Regulation of a novel gene encoding a lysyl oxidase-related protein in cellular adhesion and senescence. *J Biol Chem* 272(13), 8157-60

Shanley, C. J.; Gharaee-Kermani, M.; Sarkar, R.; Welling, T. H.; Kriegel, A.; Ford, J. W.; Stanley, J. C.; and Phan, S. H. (1997). Transforming growth factor-beta 1 increases lysyl oxidase enzyme activity and mRNA in rat aortic smooth muscle cells. *J Vasc Surg* 25, 446-52.

Scott, I. C.; Blitz, I. L.; Pappano, W. N.; Imamura, Y.; Clark, T. G.; Steiglitz, B. M.; Thomas, C. L.; Maas, S. A.; Takahara, K.; Cho, K. W.; and Greenspan, D. S. (1999) Mammalian BMP-1/tolloid-related metalloproteinases, including novel family member mammalian tolloid-like 2, have differential enzymatic activities and distributions of expression relevant to patterning and skeletogenesis. *Dev Biol* 213(2), 283-300

Selye, H. (1957) *Revue Canadienne de Biologie* 16, 3 - 82

Shimo, T.; Nakanishi, T.; Kimura, Y.; Nishida, T.; Ishizeki, K.; Matsumura, T.; and Takigawa, M. (1998). Inhibition of endogenous expression of connective tissue growth factor by its antisense oligonucleotide and antisense RNA suppresses proliferation and migration of vascular endothelial cells. *J Biochem* (Tokyo) 124, 130-40

Shimell, M.J.; Ferguson, E.L.; Childs, S.R.; O'Connor, M.B. (1991) The Drosophila dorsal-ventral patterning gene tolloid is related to human bone morphogenetic protein 1. *Cell* Nov 1;67(3):469-81

Sing, G.K.; Keller, J.R.; Ellingsworth, L.R.; Ruscetti, F.W.(1988) Transforming growth factor beta selectively inhibits normal and leukemic human bone marrow cell growth in vitro. *Blood* Nov;72(5):1504-11.

Sommer, P.; Gleyzal, C.; Raccurt, M.; Delbourg, M.; Serrar, M.; Joazeiro, P.; Peyrol, S.; Kagan, H.; Trackman, P. C.; and Grimaud, J. A. (1993). Transient expression of lysyl oxidase by liver myofibroblasts in murine Schistisomiasis. *Lab. Invest.* 69, 460 - 470.

Suzuki, N.; Labosky, P. A.; Furuta, Y.; Hargett, L.; Dunn, R.; Fogo, A. B.; Takahara, K.; Peters, D. M.; Greenspan, D. S.; and Hogan, B. L. (1996) Failure of ventral body wall closure in mouse embryos lacking a procollagen c-proteinase encoded by BMP-1, a mammalian gene related to drosophila tolloid. *Development* 122, 3587 - 95

Takahara, K.; Lyons, G. E.; and Greenspan, D. S. (1994) Bone morphogenetic protein-1 and a mammalian tolloid homologue (mtld) are encoded by alternatively spliced transcripts which are differentially expressed in some tissues. *J Biol Chem* 269(51), 32572-8

Takahara, K.; Lee, S.; Wood, S.; Greenspan, D.S. (1995) Structural organization and genetic localization of the human bone morphogenetic protein 1/mammalian tolloid gene *Genomics* Sep 1;29(1):9-15

Takahara, K.; Brevard, R.; Hoffman, G.G.; Suzuki, N.; and Greenspan, D.S. (1996) Characterization of a novel gene product (mammalian tolloid-like) with high sequence similarity to mammalian tolloid/bone morphogenetic protein-1.*Genomics* 34, 157 - 65

Tewari, D.,S.; Qian, Y.; Tewari, M.; Pieringer, J.; Thornton, R.D.; Taub, R.; Mochan, E.O.(1994) Mechanistic features associated with induction of metalloproteinases in human gingival fibroblasts by interleukin-1. *Arch Oral Biol* Aug;39(8):657-64

Tipton, D. A.; and Dabbous, M. K. (1998). Autocrine transforming growth factor b stimulation of extracellular matrix production by fibroblasts from fibrotic human gingiva. *J. Periodontol.* 69, 609 - 619.

Tipton, D. A.; Howell, K. J.; and Dabbous, M. K. (1997). Increased proliferation, collagen, and fibronectin production by hereditary gingival fibromatosis fibroblasts. *J. Periodontol.* 68, 524 - 530.

Towbin, H.; Staehelin, T.; and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350 - 4354.

Trackman, P. C.; Tang, J.; Bedell-Hogan, D.; and Kagan, H. M. (1992) Post-translational glycosylation and proteolytic processing of a lysyl oxidase precursor. *J. Biol. Chem.* 267, 8666 - 71

Trackman, P. C.; Graham, R. J.; Bittner, H. K.; Carnes, D. L.; Gilles, J. A.; and Graves, D. T. (1998). Inflammation-associated lysyl oxidase protein expression in vivo, and modulation by FGF-2 plus IGF-1. *Histochem Cell Biol* 110, 9-14.

Trojanowska, M.; LeRoy, E. C.; Eckes, B.; and Krieg, T. (1998). Pathogenesis of fibrosis: type 1 collagen and the skin. *J. Mol. Med.* 76, 266 - 274.

Uitto, V.J.; Airola, K.; Vaalamo, M.; Johansson, N.; Putnins, E.E.; Firth, J.D.; Salonen, J.; Lopez-Otin, C.; Saarialho Kere, U.; Kahari, V.M.(1998) Collagenase-3 (matrix metalloproteinase-13) expression is induced in oral mucosal epithelium during chronic inflammation. *Am J Pathol* Jun;152(6):1489-99

van der Zee, E.; Everts, V.; Beertsen, W. (1996) Cytokine-induced endogenous procollagenase stored in the extracellular matrix of soft connective tissue results in a burst of collagen breakdown following its activation. *J Periodontal Res* Oct;31(7):483-8

Vytasek, R. (1982). A sensitive fluorometric assay for the determination of DNA. *Anal. Biochem.* 120, 243 - 248.

Wardle, F.C.; Angerer, L.M.; Angerer, R.C.; Dale, L.(1999) Regulation of BMP signaling by the BMP1/TLD-related metalloprotease, SpAN. *Dev Biol* Feb 1;206(1):63-72

Williamson, M.S.; Miller, E. K.; Plemons, J.; Rees, T.; and Iacopino, A. M. (1994). Cyclosporine A upregulates interleukin-6 gene expression in human gingiva: possible mechanism for gingival overgrowth. *J Periodontol* 65, 895-903.

Wong, M.. L., K. M.; Kolesnikova, T. V.; and Lau, L. F. (1997). Cyr61, product of a growth factor-indicible immediate-early gene, regulates chondrogenesis in mouse limb bud mesenchymal cells. *Developmental Biol.* 192, 492 - 508.

Yamada, H.; Nishimura, F.; Naruishi, K.; Chou, H.H.; Takashiba, S.; Albright, G.M.; Nares, S.; Iacopino, A.M.; Murayama, Y. (2000) Phenytoin and cyclosporin a suppress the expression of MMP-1, TIMP-1, and cathepsin L, but not cathepsin B in cultured gingival fibroblasts *J Periodontol* Jun;71(6):955-60

Yamashiro T, Fukunaga T, Kobashi N, Kamioka H, Nakanishi T, Takigawa M, Takano-Yamamoto T.Mechanical stimulation induces CTGF expression in rat osteocytes. JDentRes2001Feb;80(2):461-5

Xin, L.; Martinerie, C.; Zumkeller, W.; Westphal, M.; and Perbal, B. (1996). Differential expression of novH and CTGF in human glioma cell lines. *J. Clin. Pathol.* 49, M91 - M97.

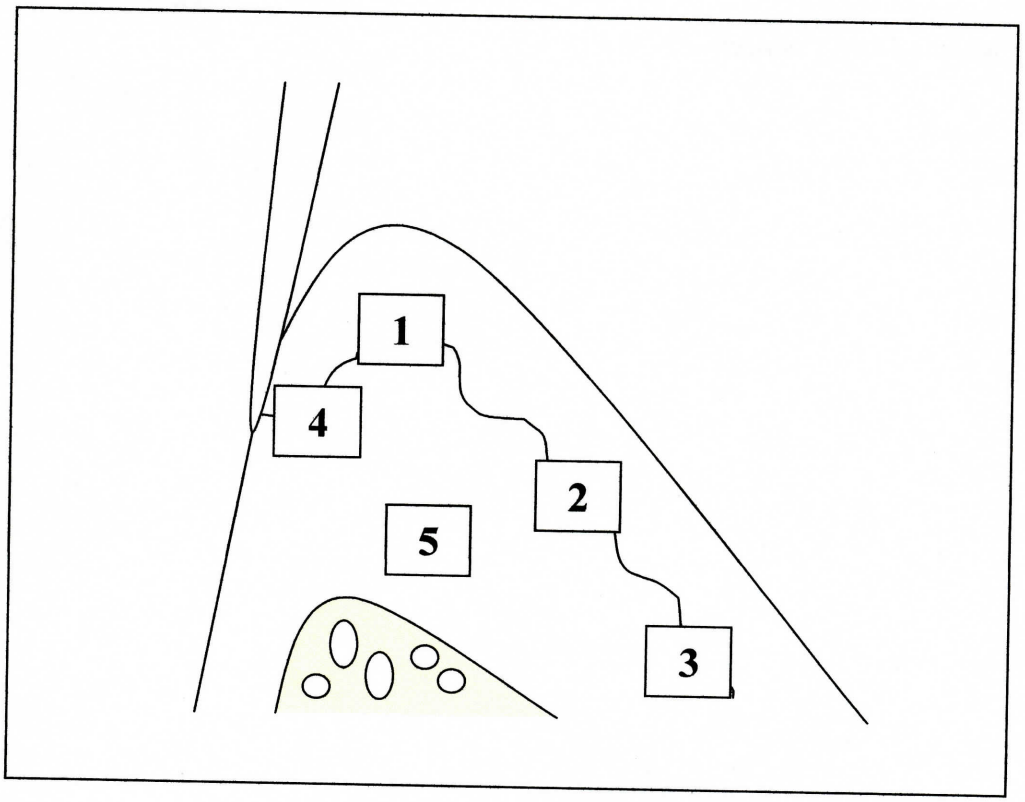

1
4
2
5
3

Figure 1 Gingival tissue areas used for evaluation of fibrosis, extracellular TGF-β1, intra and extracellular CTGF, and inflammation:

1. Oral-Sulcular Epithelium and Subepithelial Connective Tissue
2. Oral Epithelium and Subepithelial Connective Tissue
3. Oral Epithelium and Subepithelial Connective Tissue
4. Sulcular Epithelium and Subepithelial Connective Tissue
5. Middle-Deep Connective Tissue

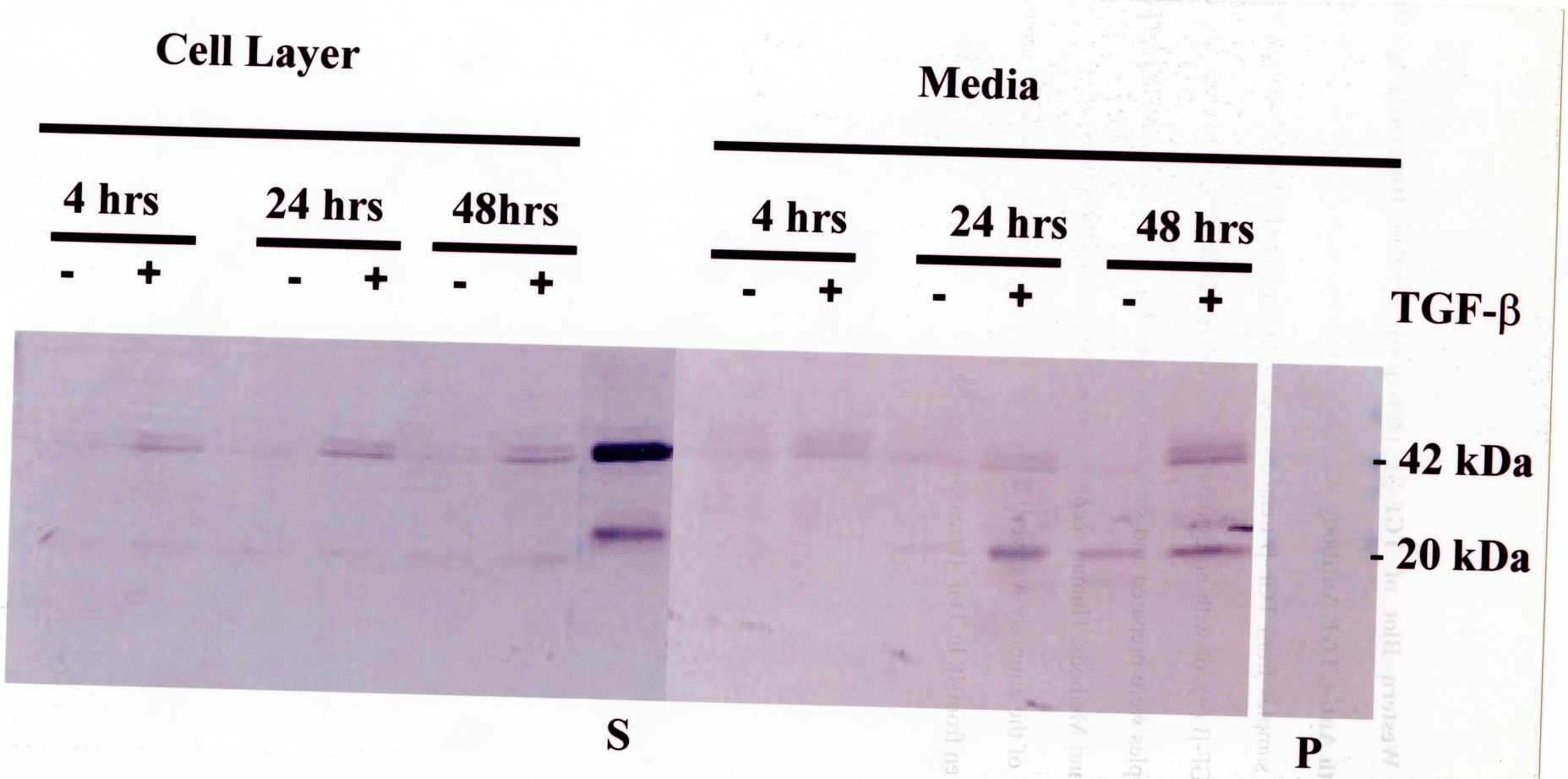

Cell Layer
Media
4 hrs
24 hrs
48hrs
4 hrs
24 hrs
48 hrs
- + - + - + - + - + - +
TGF-β
- 42 kDa
- 20 kDa
S
P

Figure 2 Western Blot of TGF-β1 (400 pM) Treated Human Gingival Fibroblasts Probed with Anti-CTGF Antibody Western blots probed with anti-CTGF antibody of cell layer and media samples from TGF-β treated human gingival fibroblasts. N5 cells were treated with 400 pM TGF-β (+), or with no cytokine (-) for the indicated periods of time, and cell layer and media samples were prepared and subjected to SDS PAGE and Western blotting as described in Materials and Methods. Human recombinant CTGF was also analyzed as a positive control (S). Specificity of the antibody was verified by lack of staining with pre-immune serum of the media sample taken from 48 hr TGF-β treated cells (P).

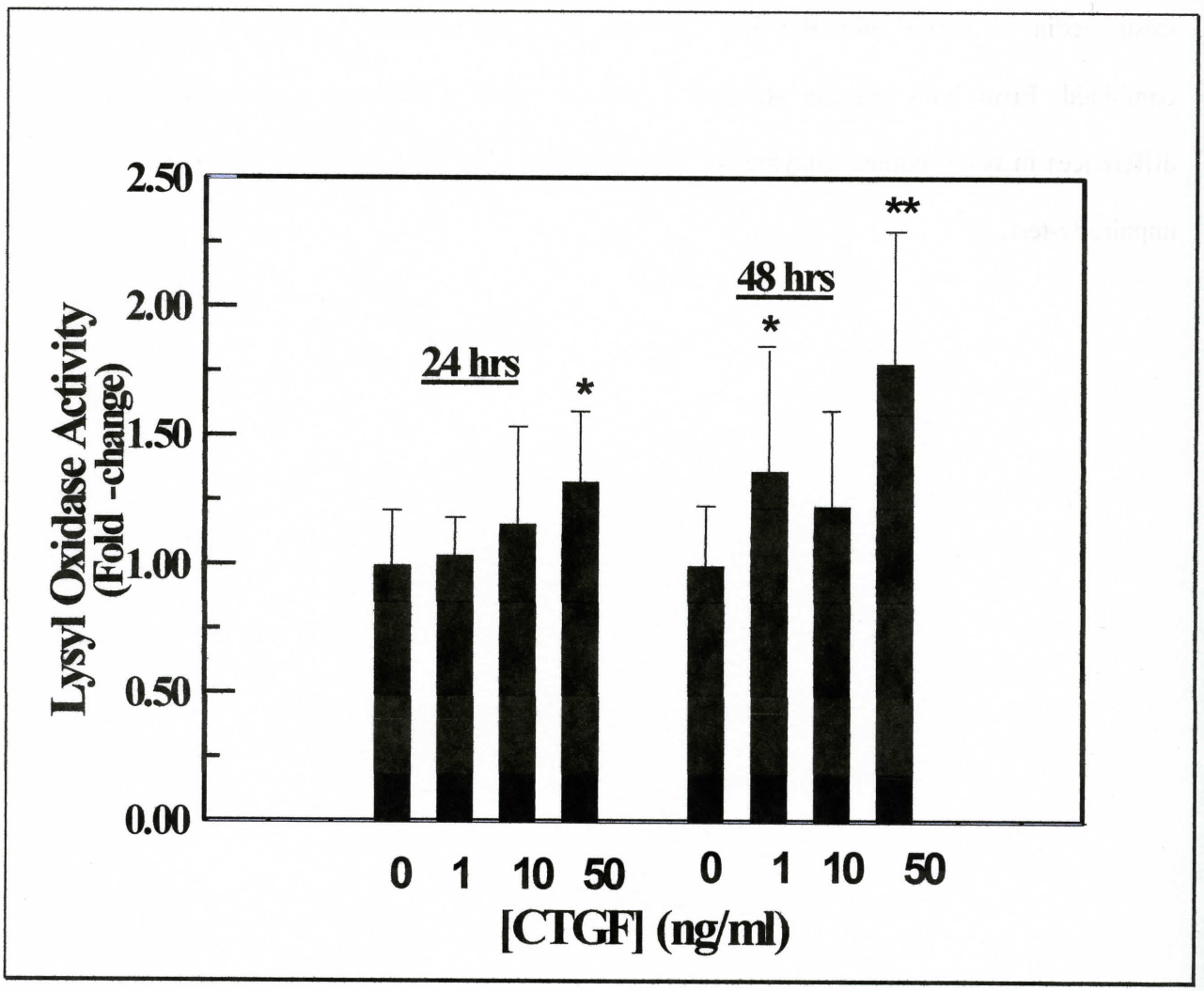

2.50
2.00
1.50
1.00
0.50
0.00
Lysyl Oxidase Activity
(Fold -change)
24 hrs
48 hrs
*
*
**
0 1 10 50 0 1 10 50
[CTGF] (ng/ml)

Figure 3 Regulation of gingival fibroblast lysyl oxidase enzyme activity by CTGF. Human recombinant CTGF was added to human gingival fibroblasts (N5 cells) at the indicated concentrations as described in "Materials and Methods", and lysyl oxidase enzyme activity/million cells was determined after 24 and 48 hours of treatment. Cells were re-fed with fresh media +/- CTGF after the first 24 hours of treatment. Data are from two experiments combined. Error bars indicate standard deviation. Asterisks indicate statistically significant differences in lysyl oxidase enzyme activity compared to untreated control cells determined by unpaired *t*-test.

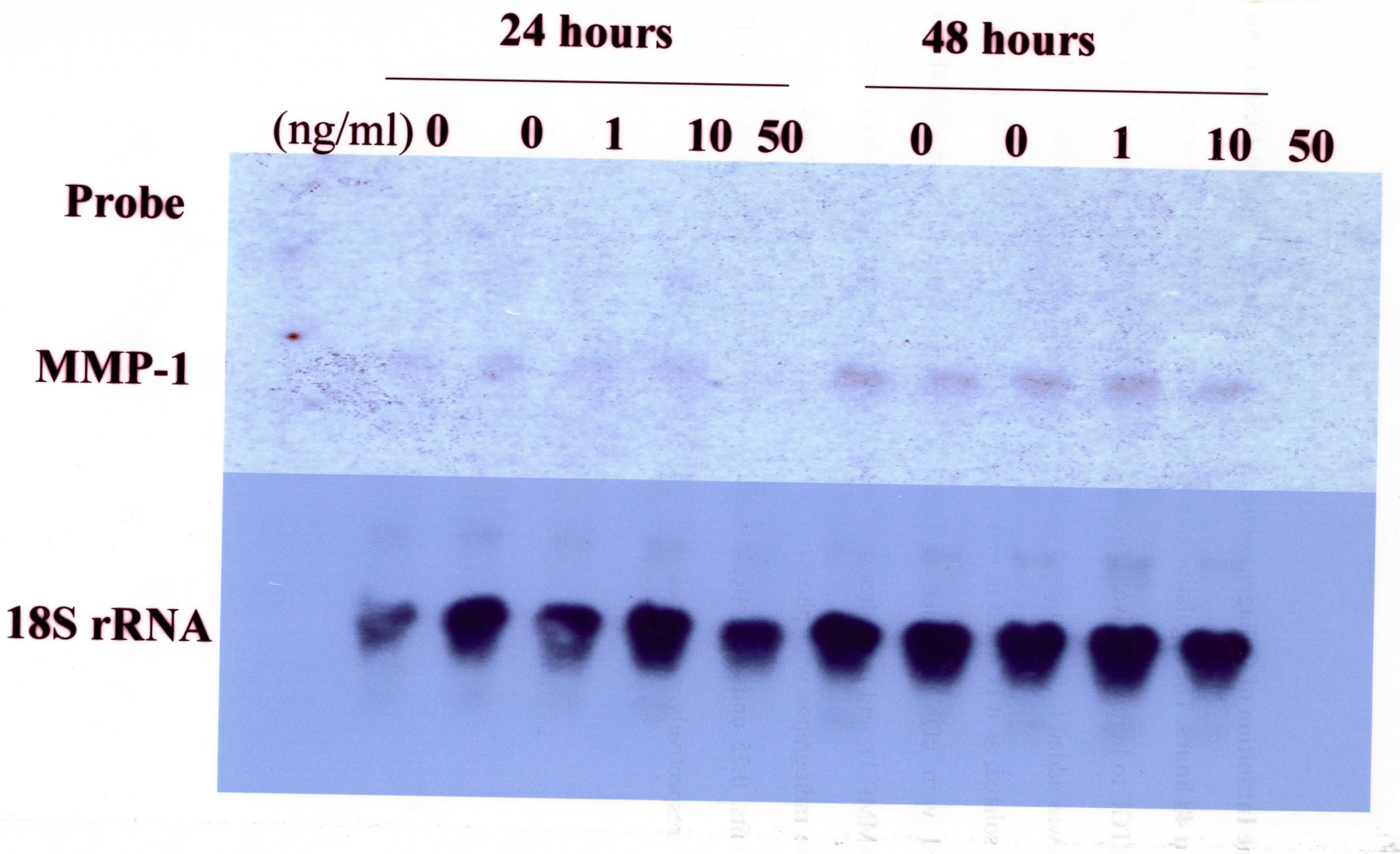

24 hours
48 hours
(ng/ml) 0 0 1 10 50 0 0 1 10 50
Probe
MMP-1
18S rRNA

Figure 4 The Inhibition of Collagenase by the Exogenous Addition of 0,10,50 ng/ml CTGF after 24 and 48 hours. The regulation of collagenase (MMP-1) mRNA levels after exogenous addition of CTGF to human gingival fibroblasts. In this evaluation, 0, 1 ng/ml, 10 ng/ml and 50 ng/ml CTGF was added to human gingival fibroblasts in serum-free medium, and 48 hours after mRNA was isolated, as it is stated in materials and methods. The Northern blots were obtained, and analyzed with cDNA probes radiolabeled utilizing random primer methodology for collagenase (MMP-1), and normalized to 18S RNA. Data were generated by evaluating optical density of the radiographs with phosphor imager. Data from the experiments, 0.72 units, 0.79 units, 0.80 units, 0.75 units of optical density after the addition, 0, 1 ng/ml, 10 ng/ml and 50 ng/ml CTGF, respectively.

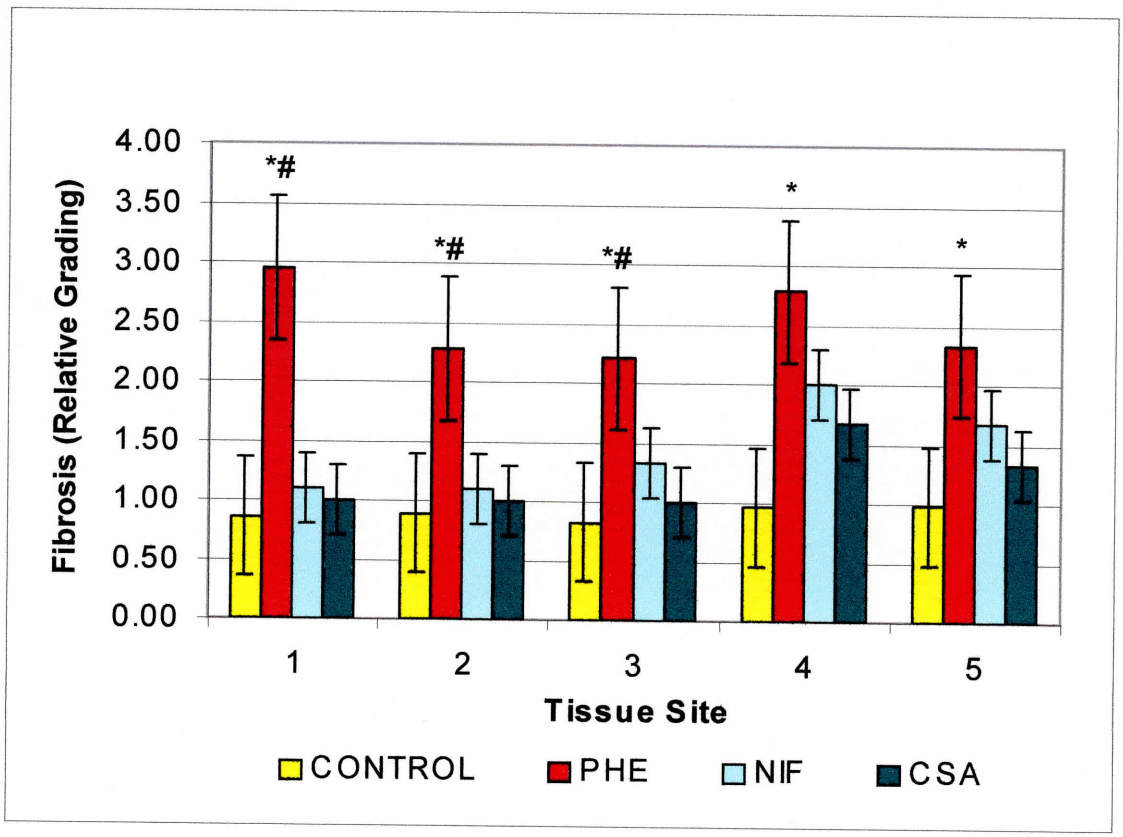

Fibrosis (Relative Grading)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*#
*#
*#
*
*
1
2
3
4
5
Tissue Site
CONTROL
PHE
NIF
CSA

Figure 5 The degree of fibrosis as assessed by the semiquantitative relative grading

The degree of fibrosis as assessed by the semiquantitative relative grading of the of collagen-like fibers and the abundance of fibroblasts (PHE: Phenytoin group, NIF: Nifedipine group, CSA: Cyclosporin-A group)

* Statistically significant ($p<0.05$) compared to CSA and C

Statistically significant ($p<0.05$) compared to NIF

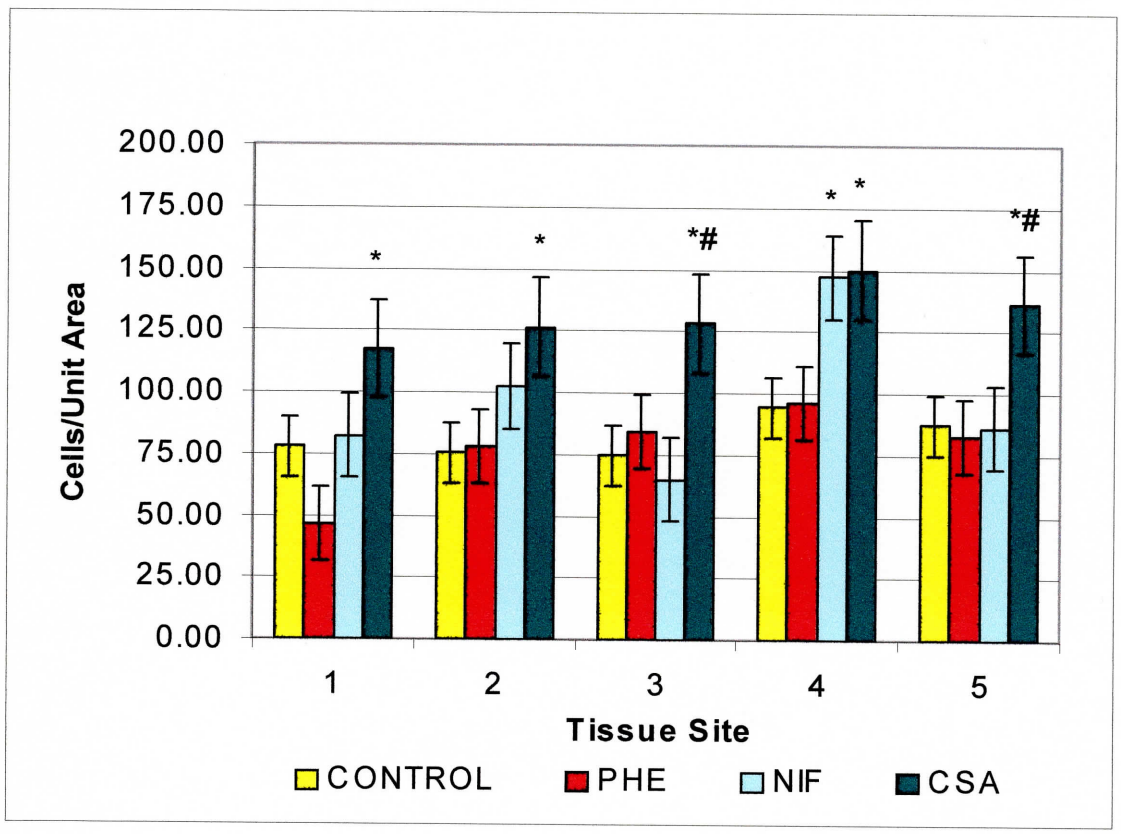
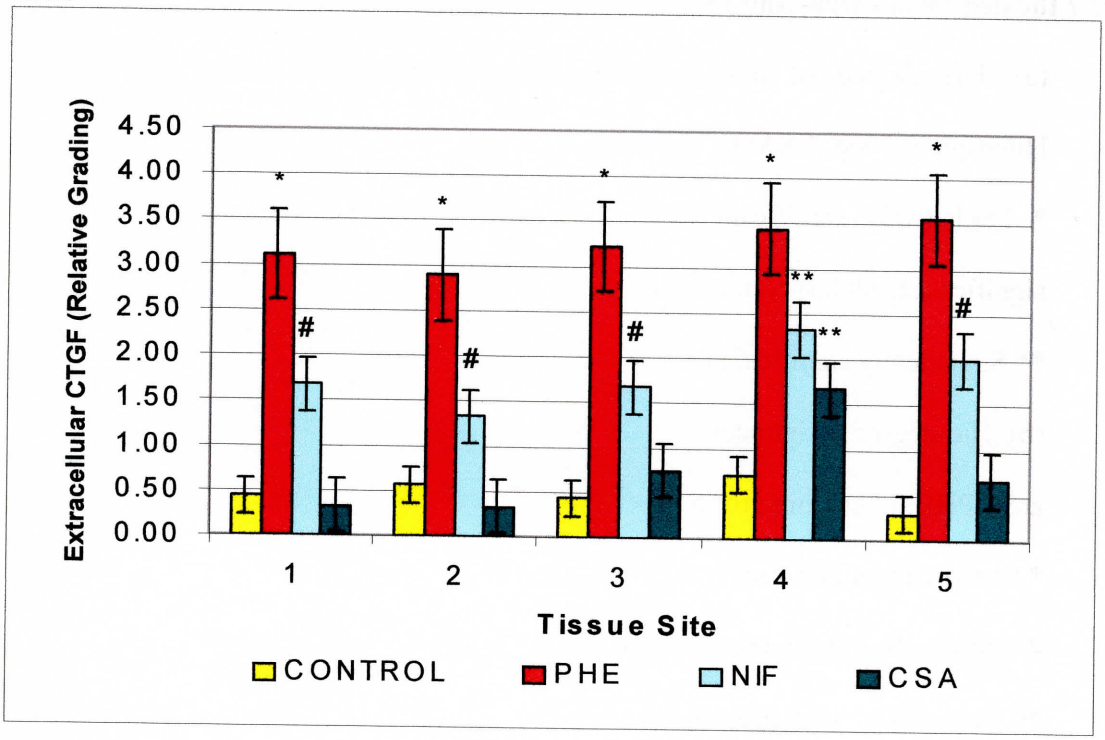

200.00
175.00
150.00
125.00
100.00
75.00
50.00
25.00
0.00
Cells/Unit Area
*
*
*#
* *
*#
1
2
3
4
5
Tissue Site
CONTROL
PHE
NIF
CSA

Figure 6. The degree of inflammation The degree of inflammation as assessed by the counts of inflammatory cells per unit area (PHE: Phenytoin group, NIF: Nifedipine group, CSA: Cyclosporin-A group) # Statistically significant ($p<0.05$) compared to NIF

Statistically significant ($p<0.05$) compared to PHE and C

a


Extracellular CTGF (Relative Grading)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
2
3
4
5
Tissue Site
CONTROL
PHE
NIF
CSA

b

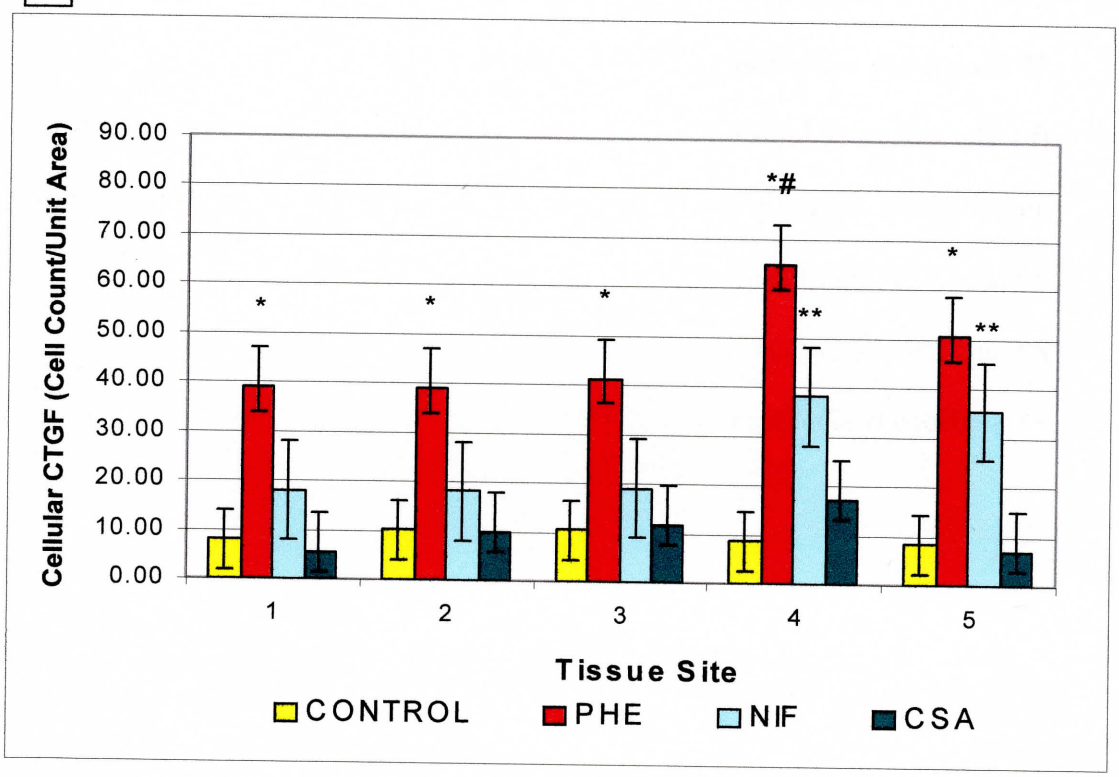

Cellular CTGF (Cell Count/Unit Area)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
1
2
3
4
5
Tissue Site
CONTROL
PHE
NIF
CSA

Figure 7 the degree of extra- and intracellular CTGF staining

(a) The degree of extracellular CTGF staining (PHE: Phenytoin group, NIF: Nifedipine group, CSA: Cyclosporin-A group)

* Statistically significant ($p<0.05$) compared to NIF, CSA, and C# Statistically significant ($p<0.05$) compared to CSA and C

** Statistically significant ($p<0.05$) compared to C

(b) The degree of intracellular CTGF staining as assessed by the cell counts per unit area (PHE: Phenytoin group, NIF: Nifedipine group, CSA: Cyclosporin-A group)

* Statistically significant ($p<0.05$) compared to NIF, CSA, and C

Statistically significant ($p<0.05$) compared to sites 1-3 and 5

** Statistically significant ($p<0.05$) compared to CSA and C# Statistically significant ($p<0.05$) compared to CSA and C

** Statistically significant ($p<0.05$) compared to C

(b) The degree of intracellular CTGF staining as assessed by the cell counts per unit area (PHE: Phenytoin group, NIF: Nifedipine group, CSA: Cyclosporin-A group)

* Statistically significant ($p<0.05$) compared to NIF, CSA, and C

Statistically significant ($p<0.05$) compared to sites 1-3 and 5

** Statistically significant ($p<0.05$) compared to CSA and C

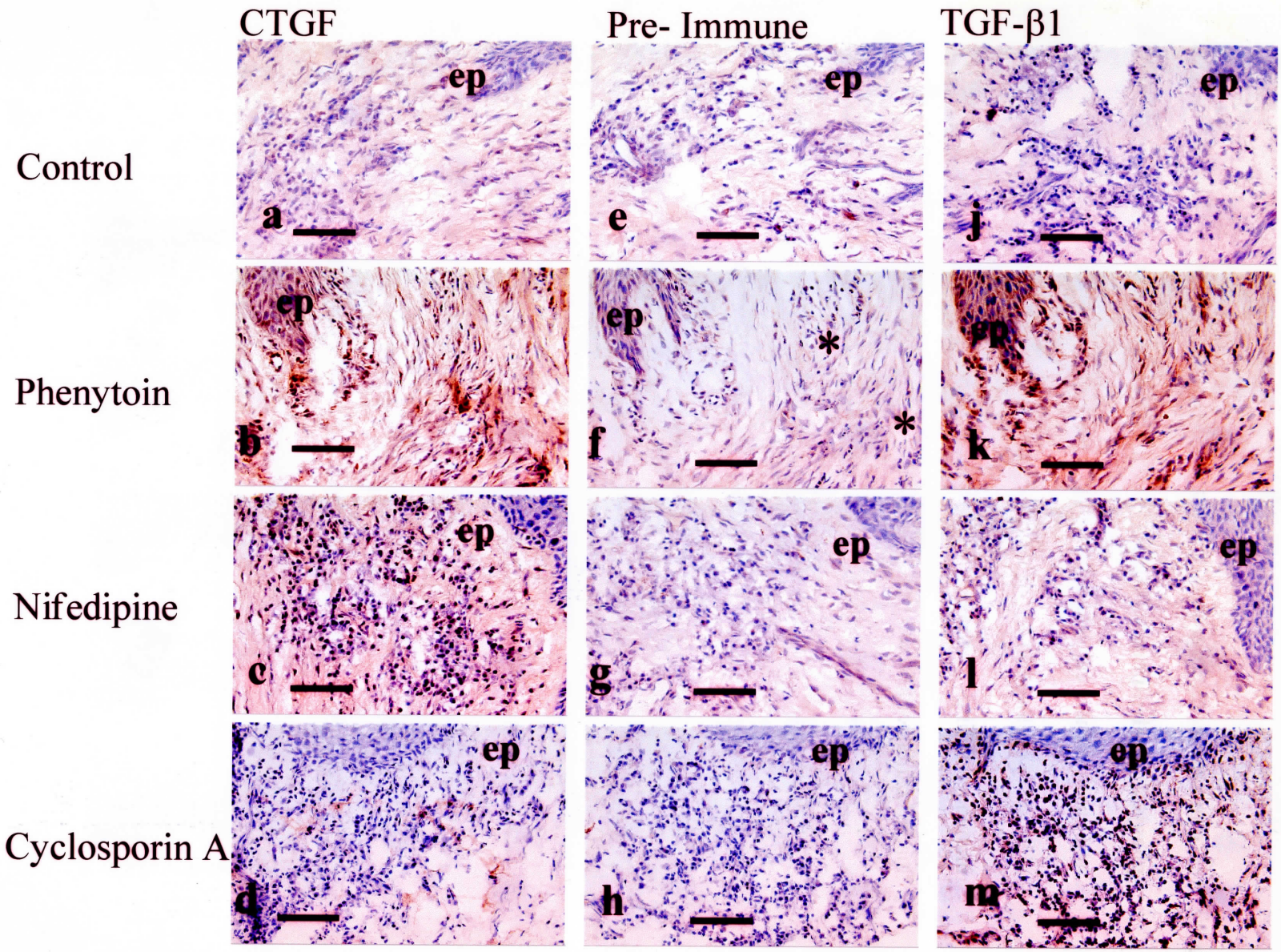
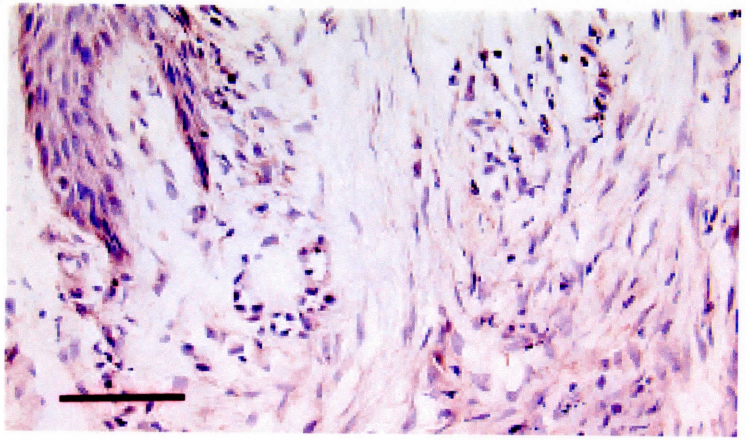
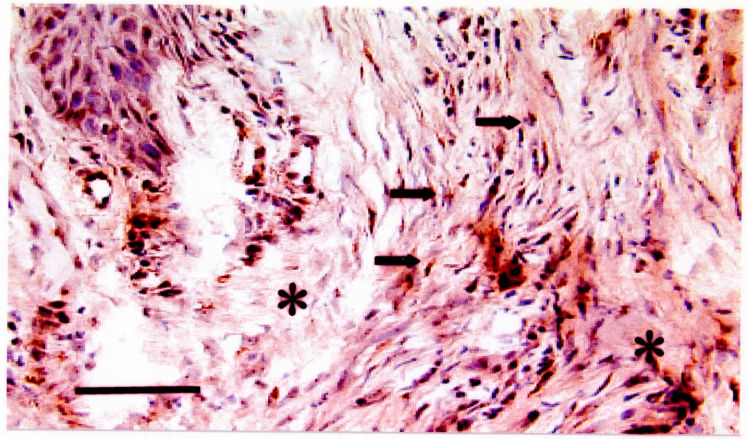

CTGF
Pre- Immune
TGF-β1
Control
Phenytoin
Nifedipine
Cyclosporin A
ep
a
b
c
d
e
f
g
h
j
k
l
m
*
*

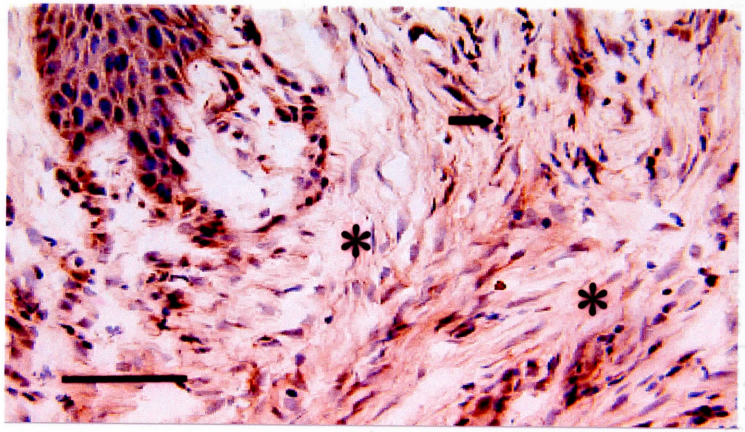
Figure 8. Comparable micrographs showing CTGF, pre-immune, TGF-β staining in phenytoin, nifedipine, cyclosporin A, and control groups Histopathology and immunohistochemical staining of gingival overgrowth tissues from phenytoin, nifedipine, cyclosporin A, and control groups with CTGF (a-d), TGFβ1 (i-l) and pre-immune staining is shown in figure e-h. "ep" stands for epithelium. (Original magnification x400; each bar=2.5μm).



Pre-Immune

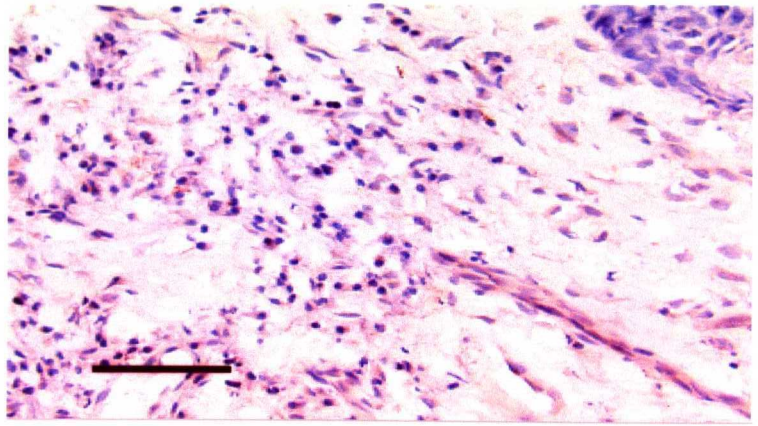


CTGF

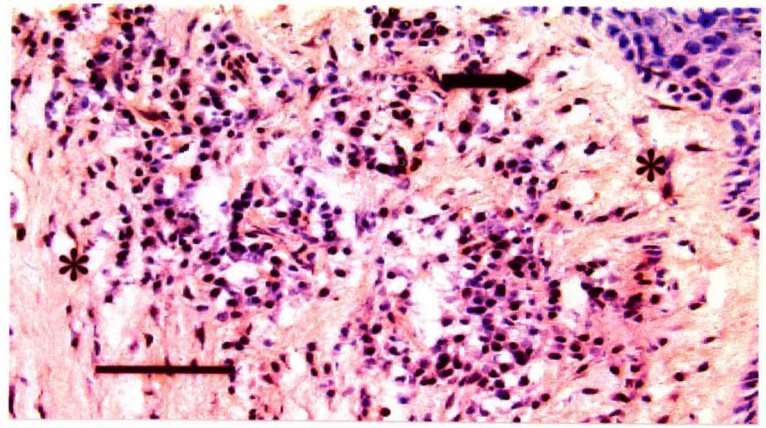


TGF-β1

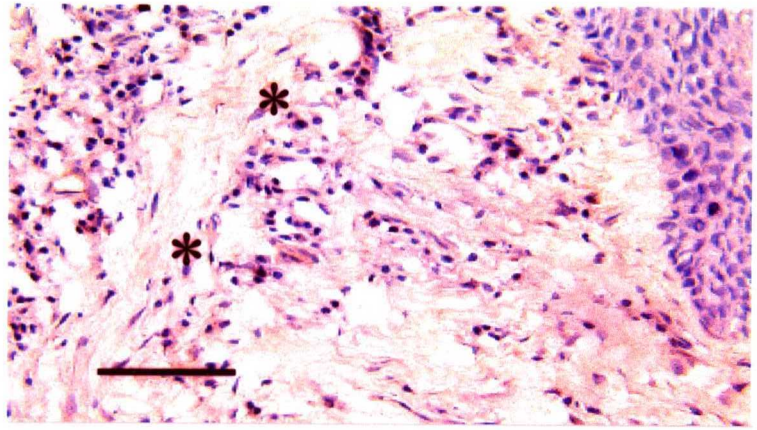
Figure 9. Representative micrographs showing CTGF, pre-immune, TGF-β staining in phenytoin group Histopathology and immunohistochemical staining of representative phenytoin-induced gingival overgrowth tissue samples with CTGF, TGFβ1 and pre-immune staining is shown. Block arrows represent intracellular staining, "*" stands for extracellular staining and clear arrows point out the blood vessels. "ep" stands for epithelium. (Original magnification x400; each bar=2.5μm).



Pre-Immune

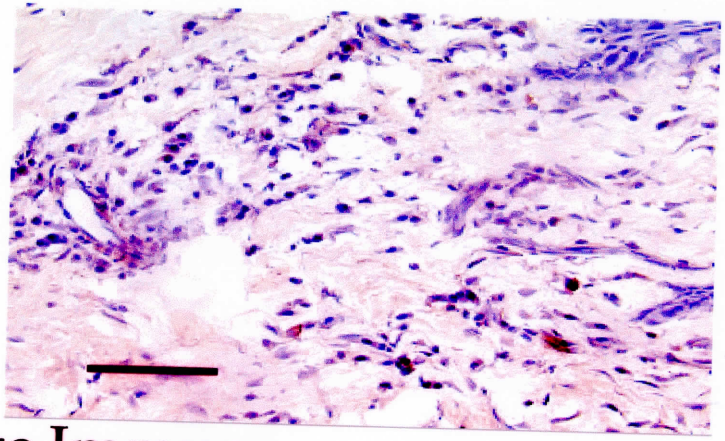


CTGF

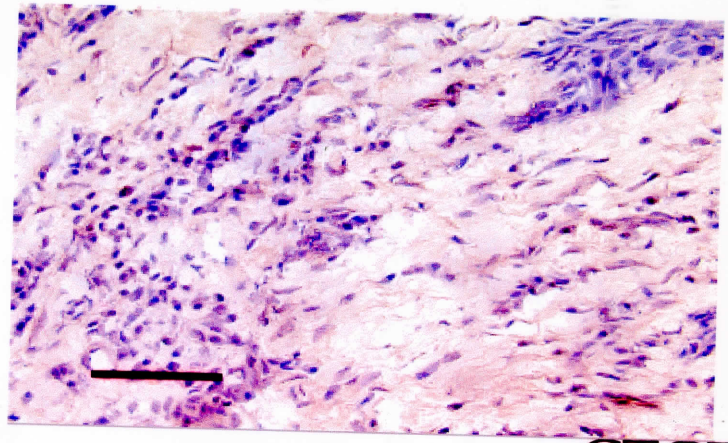


TGF-β1

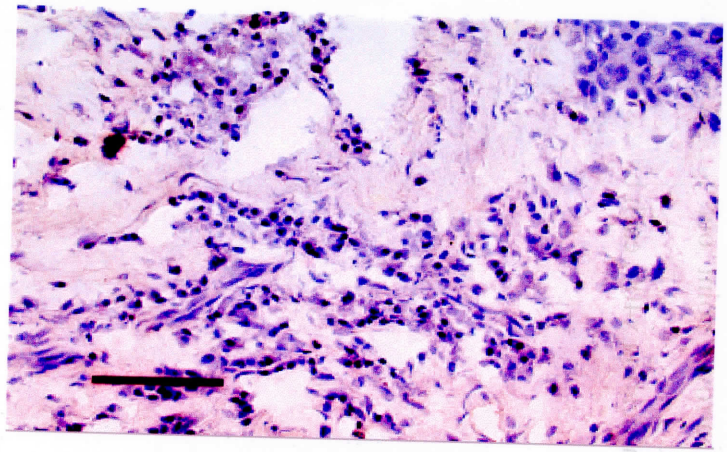
Figure 10. Representative micrographs showing CTGF, pre-immune, TGF-β staining nifedipine group Histopathology and immunohistochemical staining of representative nifedipine-induced gingival overgrowth tissue samples with CTGF, TGFβ1 and pre-immune staining is shown. Block arrows represent intracellular staining, "*" stands for extracellular staining and clear arrows point out the blood vessels. "ep" stands for epithelium. (Original magnification x400; each bar=2.5μm).



Pre-Immune

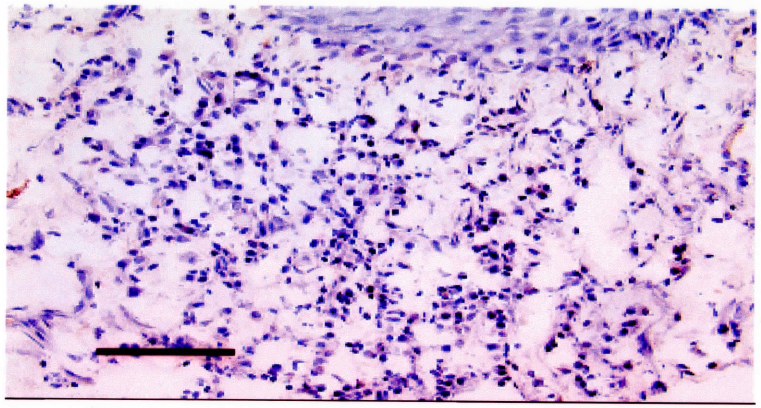


CTGF

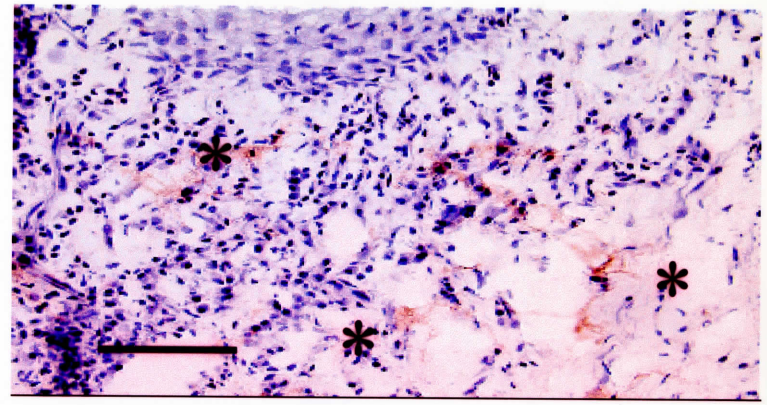


TGF-β1

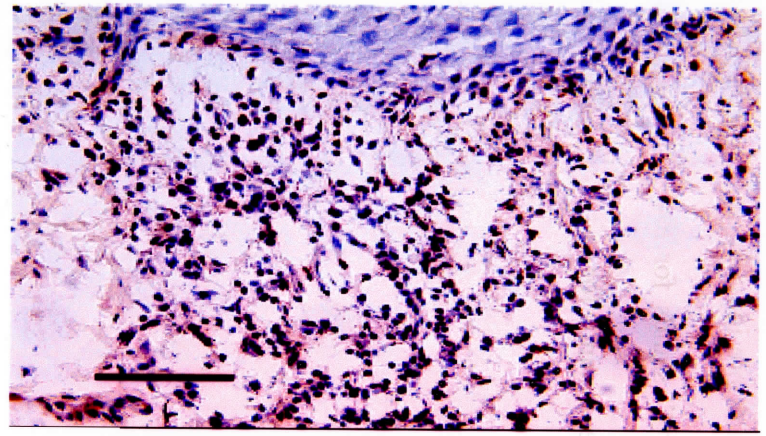
Figure 11. Representative micrographs showing CTGF, pre-immune, TGF-β staining in control group Histopathology and immunohistochemical staining of representative control group tissue samples with CTGF, TGFβ1 and pre-immune staining is shown. Block arrows represent intracellular staining, "*" stands for extracellular staining and clear arrows point out the blood vessels. "ep" stands for epithelium. (Original magnification x400; each bar=2.5μm).



Pre-Immune



CTGF



TGF-β1

Figure 12. Representative micrographs showing CTGF, pre-immune, TGF-β staining in cyclosporin A group Histopathology and immunohistochemical staining of representative cyclosporin A-induced gingival overgrowth tissue samples with CTGF, TGFβ1 and pre-immune staining is shown. Block arrows represent intracellular staining, "*" stands for extracellular staining and clear arrows point out the blood vessels. "ep" stands for epithelium. (Original magnification x400; each bar=2.5μm).

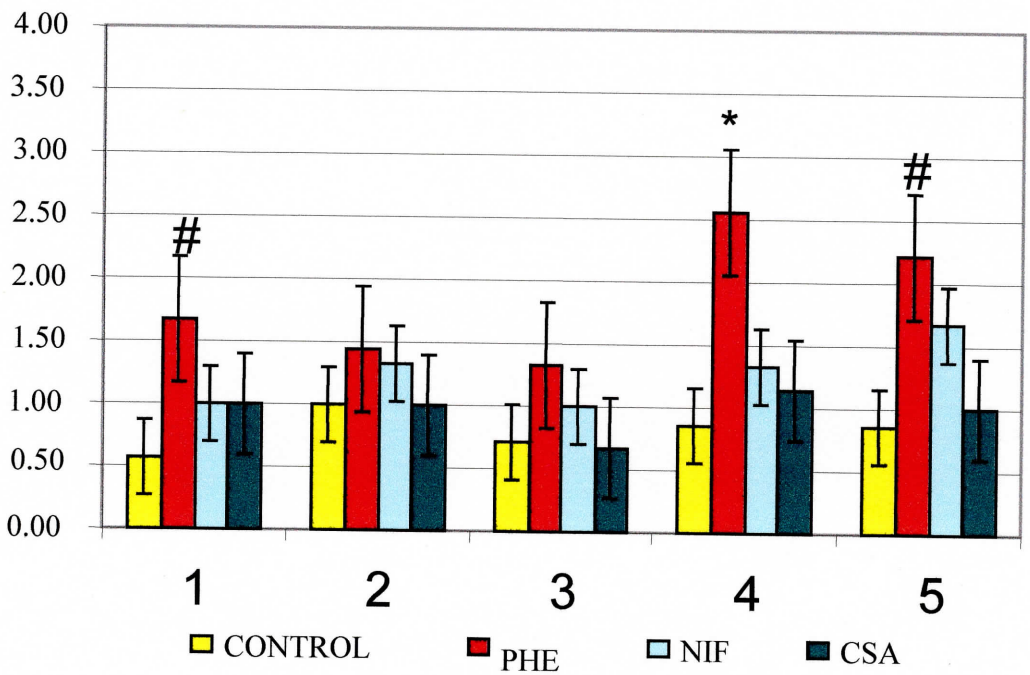

4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
#
*
#
1
2
3
4
5
CONTROL
PHE
NIF
CSA

Figure 13. The degree of relative grading of TGFβ1 staining (PHE: Phenytoin group, NIF: Nifedipine group, CSA: Cyclosporin-A group)

* Statistically significant ($p<0.05$) compared to NIF, CSA, and C

Statistically significant ($p<0.05$) compared to C

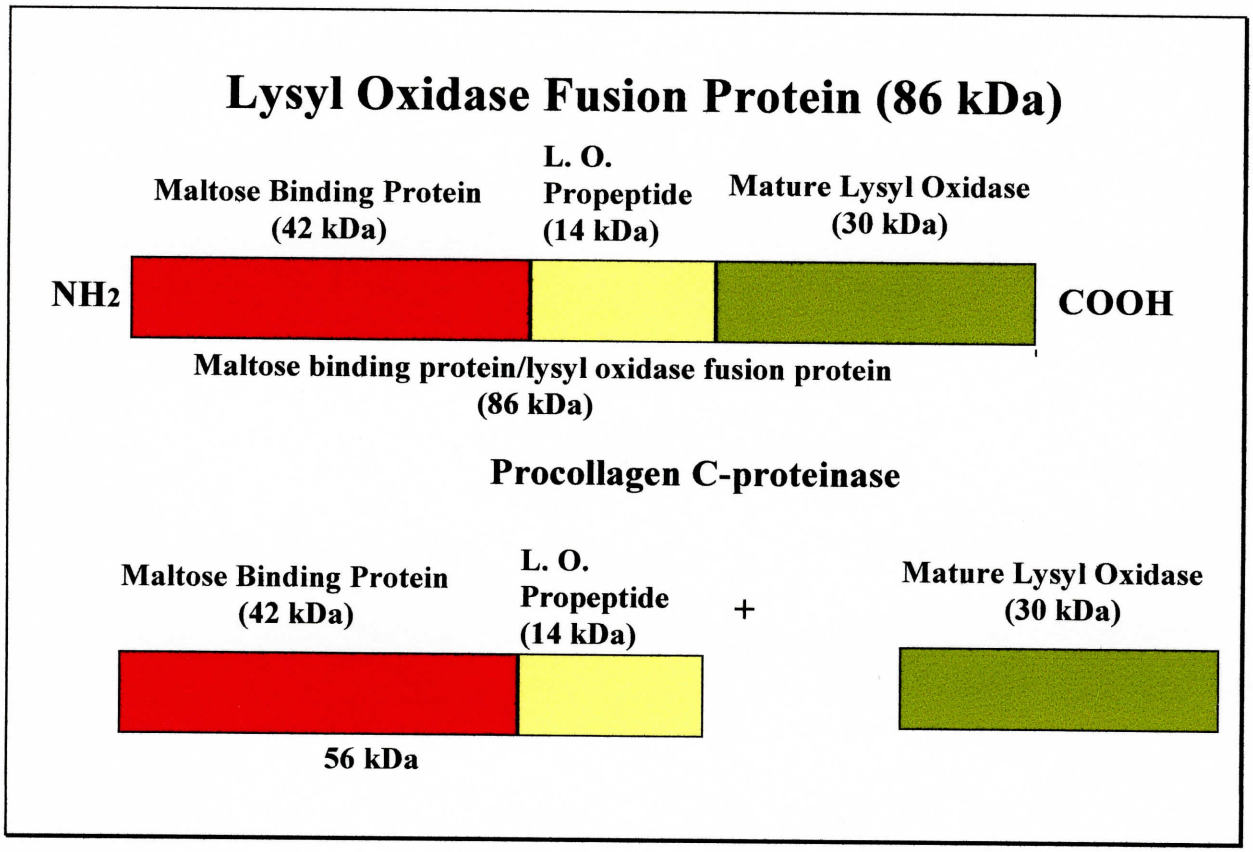
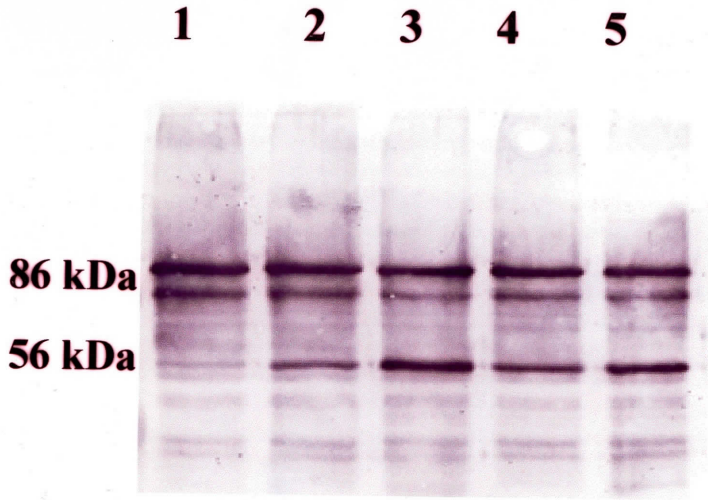
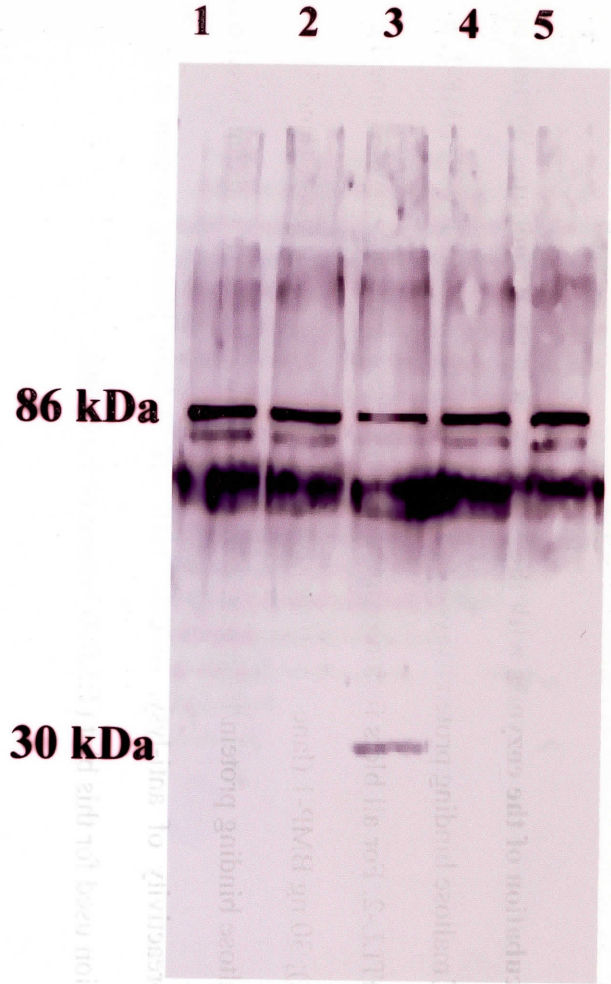

Lysyl Oxidase Fusion Protein (86 kDa)
Maltose Binding Protein
(42 kDa)
L. O.
Propeptide
(14 kDa)
Mature Lysyl Oxidase
(30 kDa)
NH2
COOH
Maltose binding protein/lysyl oxidase fusion protein
(86 kDa)
Procollagen C-proteinase
Maltose Binding Protein
(42 kDa)
L. O.
Propeptide
(14 kDa)
+
Mature Lysyl Oxidase
(30 kDa)
56 kDa

Figure 14. Maltose binding protein/lysyl oxidase fusion protein, Schematics showing the structure of the maltose binding protein/lysyl oxidase fusion protein, and products following processing by procollagen C-proteinase.



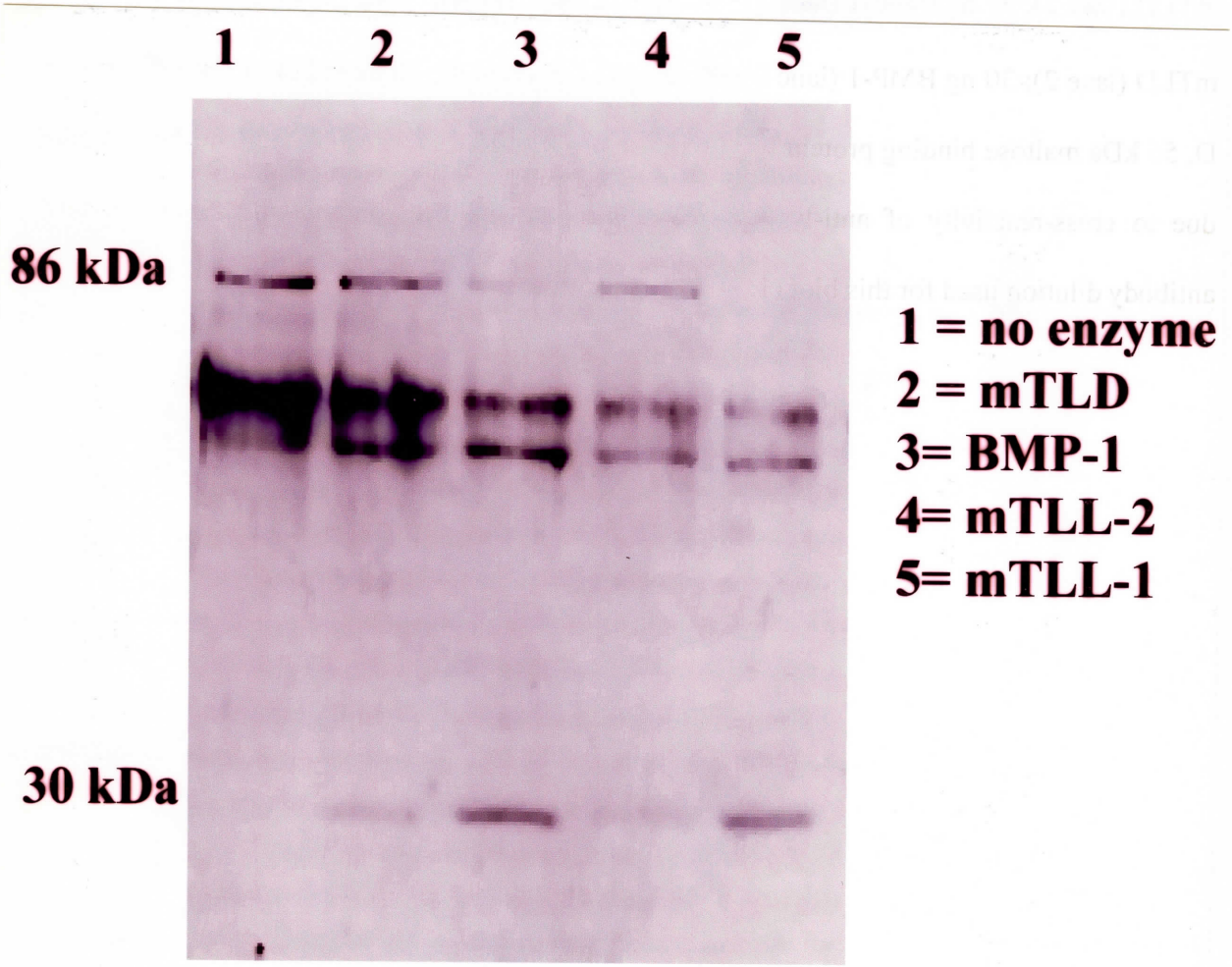
Anti-MBP

1 = no enzyme
2 = mTLD
3= BMP-1
4= mTLL-2
5= mTLL-1



Anti-LO

Figure 15. Incubation of the enzymes with the fusion protein for 45 minutes Western blots of incubations of maltose binding protein/lysyl oxidase fusion protein alone or with BMP-1, mTLD, mTLL-1, or mTLL-2. For all blots fusion protein was incubated with: no enzyme (lane 1); 30 ng mTLD (lane 2); 30 ng BMP-1 (lane 3); 30 ng mTLL-2 (lane 4); 30 ng mTLL-1 (lane 5). In panel D, 56 kDa maltose binding protein/lysyl oxidase propeptide was detected with anti-lysyl oxidase due to cross-reactivity of anti-lysyl oxidase with the maltose binding protein at the lower antibody dilution used for this blot (1:5,000 instead of 1:10,000).



Anti-LO

Figure 16. Incubation of the enzymes with the fusion protein for 4 hours Western blots of incubations of maltose binding protein/lysyl oxidase fusion protein alone or with BMP-1, mTLD, mTLL-1, or mTLL-2. For all blots fusion protein was incubated with: no enzyme (lane 1); 30 ng mTLD (lane 2); 30 ng BMP-1 (lane 3); 30 ng mTLL-2 (lane 4); 30 ng mTLL-1 (lane 5). In panel D, 56 kDa maltose binding protein/lysyl oxidase propeptide was detected with anti-lysyl oxidase due to cross-reactivity of anti-lysyl oxidase with the maltose binding protein at the lower antibody dilution used for this blot (1:5,000 instead of 1:10,000).

Day of Harvest	Collagen/DNA (μg/μg) (mean +/- SE)	
	No CTGF	+ 50 ng/ml CTGF
0	4.96 +/ 2.5	5.94 +/- 3.0
4	10.5 +/- 0.12	15.35* +/- 0.69
11	15.34 +/- 0.67	31.58* +/- 1.52
18	17.54 +/- 0.32	26.44* +/- 0.96

Table I. Stimulation of cell layer collagen levels in gingival fibroblasts by 50 ng/ml CTGF.

Human gingival fibroblasts (culture N5) were grown to confluence in DMEM containing 10% NBS and antibiotics. Cells were then cultured in this medium containing in addition 100 μg/ml ascorbate (day 0). Treatment with CTGF (50 ng/ml) or no CTGF was begun on day zero. Media were changed three times per week. Cell layers were harvested after washing with PBS by scraping into 0.01% Triton-X 100 with a rubber policeman. Aliquots were used for collagen determination calculated from hydroxyproline levels measured in triplicate by amino acid analysis, and for DNA determination (Vytasek, 1982) (see "Materials and Methods"). This experiment was performed twice with similar results.

* Statistically greater than control (no CTGF) by unpaired t-test ($p<0.05$).