Analysis of Cells and Matrix in Cyclosporine-induced Gingival Overgrowth

By:

Masoumeh Nouri

DDS, Shahid Beheshti University of Medical Sciences 1985
Dip. Perio, Shahid Beheshti University of Medical Sciences 1989

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Abstract

- Background and objective: Gingival overgrowth is one of the side effects of cyclosporine A, an immunosuppressant agent. Cyclosporine A is used clinically to counteract rejection following organ transplantation. The aim of the present study was to characterize the tissue changes that occur in gingival tissue in patients receiving this medication, compared to control patients' tissues.

-Materials and Methods: Gingival biopsies were taken from 18 kidney transplant patients referred to Labafi hospital in Tehran/ Iran. Periodontal flap approach was a part of the procedure to treat their gingival overgrowth. In order to compare these samples with normal tissues, 12 gingival tissue samples were obtained from healthy individuals in the process of crown lengthening that took part in the school of dentistry in SHB University, Tehran/ Iran. The patients from the two groups were as sex and age-matched as much as possible. In all cases, patients gave informed consent for the use of their tissue samples for research. Tissue collection, fixation, histological and histochemical stainings were performed under the same conditions in both groups. The antibodies utilized for immunohistochemical stainings comprised of 1A4, PCNA, LC2, 2B1 and CD44. These antibodies target α-smooth muscle actin, proliferating cells, c terminal and core of versican and hyaluronan receptors, respectively.

-Results: Compared to controls, histological features from gingival overgrowth samples revealed changed retepegs, some degrees of acanthosis, hyperkeratosis and parakeratosis, increased vascularization, and severe inflammatory infiltration. The inflammatory infiltrate included high number of macrophages. Matrix changes included prominent accumulation of glycosaminoglycans (GAGs) and proteoglycans (mainly versican).

Conclusion: Both epithelium and connective tissue showed changes in gingival overgrowth samples. Gingival overgrowth tissues showed increased inflammation in which CD44 positive cells (macrophages) predominated. More proliferative activity and changes in retepegs were observed in epithelium. The presence of myofibroblasts was noted in connective tissue. Versican accumulation was also observed in connective tissue, in gingival overgrowth samples. Our studies are consistent with a macrophage-driven accumulation of versican-rich connective tissue, associated with epithelial proliferation in gingival overgrowth, secondary to cyclosporine therapy.

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"Love is the root of all."
(Ostad Elahi 1895-1974)

Chapter One- Introduction

1- Normal Gingiva:

1-1 Macroscopic Anatomy:

The gingiva is that part of the masticatory mucosa which covers the alveolar process and surrounds the cervical portion of the teeth (Lindhe *et al* 2003). The gingiva is divided anatomically into marginal, attached and interdental areas (Newman *et al* 2002).

1-2 Microscopic Anatomy:

The gingiva consists of a central core or connective tissue covered by stratified squamous epithelium. The epithelium is divided into four cell layers: basal, prickle cell or spinous, granular and keratinized cell layer. Epithelium can be orthokeratinized, parakeratinized or non-keratinized. Orthokeratinized epithelium has no nuclei in the keratinized cell layer and the granular cell layer is well-defined. In parakeratinized epithelium, the keratinized cell layer contains pyknotic nuclei. Non-keratinized epithelium has neither granular nor keratinized cell layer. The superficial cells in this type of epithelium have viable nuclei. The principal cell type of the gingival epithelium is the keratinocyte. The keratinocytes undergo cell division in the basal cell layer and then migrates across the epithelium to reach the superficial layers. Other cells found in the epithelium are melanocytes, langerhans cells, merckle's cells and inflammatory cells. These cells are also called clear cells. The boundary between the epithelium and the connective tissue has a wavy course. The connective tissue portions which project into the epithelium are called connective tissue papillae and are separated from each other by retepegs. The epithelium is joined to the connective tissue by a basal lamina consists of lamina densa and lamina lucida by electron microscopy. The major components of the connective tissue are collagen fibers, fibroblasts, vessels and nerves which are embedded in an amorphous ground substance. Collagen type I forms the bulk of the connective tissue. Type IV collagen is found in the basement

membranes. Elastic fibers are also distributed among collagen fibers. Fibroblasts synthesize collagen and elastic fibers as well as the glycoproteins and glycosaminoglycan-containing proteoglycans of the amorphous intercellular matrix. Besides fibroblasts, the connective tissue also harbors mast cells and macrophages as well as inflammatory cells (Newman *et al* 2002, Lindhe *et al* 2003).

2-Gingival overgrowth:

2-1 Definition:

Gingival overgrowth or gingival enlargement is the increase in size of the gingiva, which is a common feature of gingival disease. These are both clinical descriptive terms that avoid the erroneous pathological connotations of terms used in the past such as gingival hyperplasia. Different types of gingival enlargement are classified according to etiologic factors and pathologic changes such as; inflammatory enlargement, drug-induced enlargement, enlargements associated with systemic diseases and conditions and neoplastic enlargements (Carranza & Hogan 2002). In addition to cyclosporine A, phenytoin and nifedipine are the two other major medications which can cause gingival overgrowth. All of these drugs function by inhibiting the cellular uptake of calcium (Lindhe *et al* 1998).

2-2 Prevalence and clinical features of cyclosporine-induced gingival overgrowth:

Gingival overgrowth is observed initially as a papillary enlargement which is prominent on the labial aspects. The enlargement coalesces, resulting in a lobulated appearance (Tyldesley *et al* 1984). Gingiva has been reported to be erythematous, edematous, mobile, tender and tending toward spontaneous hemorrhage in some cases (Rostock *et al* 1986). Enlarged tissues associated with CSA treatment are generally more hyperemic than the other medications- induced gingival overgrowth. The first cases of gingival overgrowth caused by CSA medication were reported by Rateitschak *et al* in 1983. These authors studied 50 kidney transplant patients, most of whom developed gingival

enlargement after 4-6 weeks of CSA treatment. However, it has also been reported by several authors that gingival overgrowth starts over several months, usually between the first and third month after transplantation and often reaches a plateau after 1 year of CSA therapy (Hallmon & Rossmann 1999, Afonso *et al* 2003). CSA overgrowth seldom occurs at sites distant from teeth or in edentulous patients (Marshall & Bartold 1999). Because of this finding, Fu *et al* (2001) carried out an animal study to examine overgrowth occurrence at the edentulous ridge after CSA therapy in rats. They extracted all right maxillary molars in 16 rats and separated animals into 2 groups, a control and a CSA-treated group. The edentulous morphology, including the bucco-lingual width and the vertical height was measured after sacrificing the animals. Results showed that CSA therapy produced a significant increase of the ridge width and height, when compared to controls. Under histometry, CSA treatment resulted in a significant increase of the epithelium, connective tissue and total soft tissue areas. Based on the results of this study, the authors questioned the hypothesis that tooth or PDL is an essential component for the overgrowth development. The reported incidence for CSA-induced gingival overgrowth ranges from 13% to 81% (Afonso *et al* 2003).). In other studies the prevalence was determined to range from 25% to 50% (Hallman & Rossmann 1999). The reasons for this extensive range are the nature of the disease being treated, the age of the patient, the method of measuring gingival overgrowth and additional medications being taken by the patient (Marshall & Bartold 1999, Afonso *et al* 2003). Some authors found a positive correlation between CSA blood concentration and incidence of overgrowth (Marshal & Bartold 1999). Hefti *et al* (1994) in a double blind clinical trial reported that 17% of those on CSA with blood levels below 400 ng/ml developed gingival overgrowth, whereas 59% taking CSA with blood levels above 400 ng/ml developed enlarged gingiva.

Many studies have reported no significant correlation between the occurrence of gingival overgrowth and duration of CSA treatment (McGaw *et al* 1987, Pernu *et al* 1992, Cebeci *et al* 1996, Cakir *et al*

1998, Afonso *et al* 2003). There are also some additive effects reported when CSA is administered concurrently with nifedipine (NIF).Thomason and Seymour (1993) compared 32 renal transplant patients who were medicated with CSA for at least 3 months with 23 similar patients who were medicated with both CSA and NIF. Patients on combined treatment demonstrated a significantly higher gingival overgrowth score when compared to the patients on CSA. The prevalence of gingival overgrowth was 48% for the combined group and 37% for the CSA group. The same authors in another study assessed the gingival health of cardiac transplant patients who were on cyclosporine or a combination of CSA and NIF. 62% of those taking combined treatment and 26% of the patients only on CSA exhibited gingival overgrowth. They found that there was a 25-fold increased risk of requiring surgery if a patient was taking both medications. They also reported that young patients are more susceptible to drug induced gingival overgrowth as well as noting tissue changes to be more prevalent in males (Thomason & Seymour 1995).

2-3 Clinical complications:

As the overgrowth becomes more pronounced, there are increased esthetic concerns. In addition to esthetic problems, the overgrowth may result in uncleansible areas that are more prone to infection, caries, and the development of periodontitis (Williamson *et al* 1994). Gingival overgrowth also may adversely affect the ability to masticate (Rostock *et al* 1986).

One recent study (Boratynska *et al*, 2003) demonstrated a higher frequency of loss of graft function due to chronic graft nephropathy among patients treated with CSA who suffer from gingival overgrowth. No research on a possible correlation between gingival hyperplasia and chronic graft nephropathy has been performed to date. However, based on the above mentioned study, there are common pathological mechanisms for both kidney fibrosis and gingival overgrowth. The authors

stated that TGF-β may be one factor responsible for this pathogenesis. Based upon the findings of this study, we can conclude that gingival overgrowth is of clinical significance, not only for the oral complications it causes, but also it can be a sign showing a higher risk of nephropathy.

3-Cyclosporine A:

3-1 History:

The discovery of cyclosporine A (CSA) is attributed to Jean Borel (Alagille 1994). Its first reported use in renal transplant was by Calne *et al* (1978). Although it was first isolated in Switzerland in 1970 as a metabolite of a fungus species, it proved to have little value as an antifungal antibiotic. However, as a potent immunosuppressive agent, it prolongs survival of allogeneic transplants (Hallmon & Rossmann 1999).

3-2 Mechanism of Action:

CsA suppresses some humoral immunity but is more effective against T-cell-dependent immune mechanisms such as those underlying transplant rejection and some forms of autoimmunity. It preferentially inhibits antigen-triggered signal transduction in

T lymphocytes, blunting expression of many lymphokines, including IL-2, as well as expression of anti-apoptotic proteins. Studies on the biological mechanisms of CSA have shown that the CSA-cyclophilin complex binds and inhibits the activation of calcineurin, a calcium and camodolin dependent serine threonine phosphatase. This inactivation prevents dephosphorylation of nuclear factor of activated T cells (NF-AT), and thereby the nuclear import of NF-AT and the formation of a transcriptionally active NF-AT complex. The net consequence, inhibition of IL-2 gene expression at the transcriptional level, is considered to be the primary mechanism for the immunosuppressive

activity of CSA (shin *et al* 1997). CSA can inhibit some *in vitro* B cell responses to T cell independent antigens but the clinical significance of this is unclear (O'Garra *et al* 1986).

3-3 Disposition and Pharmacokinetics

CSA is variably absorbed in the gut; peak plasma concentration is reached 3-4 h after administration, it also has a variable serum half-life (17-40 hours). However there is a high variability in the data reported on the terminal half-life of CSA depending on the assay applied and on the target population (CPS 2001).

Bioavailability and time until the serum concentration peaks vary greatly between individuals (Marshall & Bertold 1998). The drug is mostly bound to both cells (erythrocytes 50%, lymphocytes 5%) and lipoproteins (40%), with approximately 5% free in the plasma (Hassell & Hefti 1991). CSA accumulates in the pancreas, spleen, liver, fat, kidney, lung, bone marrow, heart and whole blood (Atkinson *et al* 1982). The total concentration of the drug per weight in tissue can be up to 20-fold greater than in plasma and varies considerably between organs and individuals. CSA is metabolized in the liver microsomes; in particular by members of the cytochrome P-450-IIIA gene family, which is also responsible for the metabolism of hydrophobic compounds such as nifedipine (Yatscoff *et al* 1991). Another cytochrome, P-450 hPCN3, has also been shown to be involved in metabolizing CSA (Aoyama *et al* 1989). These monooxygenase enzymes produce at least 15 metabolites, which are not as immunosuppressive as the parent compound. CSA is excreted mainly via the bile, through the faeces. There is only 6% excreted in the urine with only 0.1% excreted in urine as unchanged drug (Masshall & Bartold 1998, CPS 2001).

3-4 Therapeutic Uses

The success achieved with CSA treatment in transplant medicine and a wide variety of autoimmune diseases allows us to estimate that one billion persons world wide will be taking CSA in the next decade (Iacopino et al, 1996). The main indication for this medication is solid organ transplantation. CSA in form of Neoral capsules and oral solution and Sandimmune I.V. are indicated in the prevention of graft rejection following transplantation (Hallmon, Rossmann 1999 and CPS 2001). Other indications include bone marrow transplantation, psoriasis, rheumatoid arthritis, nephritic syndrome (Marshal & Bartold 1998, Hallmon & Rossmann 1999, CSP 2001), Behcet's syndrome (Pisanty *et al* 1987), insulin-dependent diabetes mellitus and systemic lupus erythematosus (Rostock *et al* 1986).

3-5 Side Effects:

Several side effects of CSA treatment have been reported, among which are chronic nephrotoxicity, hepatotoxicity and neurotoxicity, lymphoproliferative neoplasms, hypertension, thromboembolic complications, biliary calculus disease, diabetes, altered bone metabolism, hirsutism, cutaneaous disorders, high risk of opportunistic infections and finally gingival overgrowth (Marshall & Bartold 1998, Rostock *et al* 1986, Rateitschak *et al* 1983). Nephrotoxicity is a common complication of CSA therapy. It occurs as CSA causes increases in serum creatinine and urea levels as a result of reduced glomerular filtration rate, even at recommended doses. Cyclosporine also might cause a reversible increase in serum bilirubin and liver enzymes, leading to hepatotoxicity. With regard to these side effects, close monitoring of renal and hepatic function is required for patients treated with CSA. CSA also has the potential to induce tremor, convulsion and paresthesia. Malignancies and lymphoproliferative disorders can develop as well, but their incidence and distribution are similar to

those in patients treated with conventional immunosuppressive therapy (CPS 2001). Gingival overgrowth is a side effect of CSA that is less damaging to the patient's health. However, considering its high incidence and the clinical complications that it causes, it is a significant side effect for the patients' quality of life (Afonso *et al* 2003). Studies addressing the mechanism of gingival overgrowth in a rat model, demonstrated that CSA immunosuppression inhibits the activity of matrix metalloproteinase 2 and 9 in the early phase of granulation tissue in healing dental socket (Silva *et al* 2001) Based on these findings, the authors suggested that CSA may interfere with the wound healing following dental extractions. However, more clinical studies are needed to determine whether CSA interferes with the tissue healing.

4-Pathogenesis of CSA-induced gingival overgrowth:

Studies of the pathogenesis of CSA-induced gingival overgrowth on the cellular and extracellular components of periodontal tissues have produced considerable data. It seems that complex interactions between various mediators of inflammation and tissue modeling are involved in the gingival overgrowth.

4-1 Cells:

-Macrophages:

There have been a number of studies of macrophage subpopulations in gingival overgrowth induced by cyclosporine and nifidipine, Nurmenniemi *et al* (2002) studied gingival samples taken from individuals on CSA and nifedipine treatment and compared those to samples taken from healthy individuals. Antibodies used for immunohistochemical staining were 27E10, RM3/1 and 25F9. 27E10 antibody identifies a type of macrophages found in cases of acute inflammation. RM3/1 antigen is

expressed by a reparative/proliferative macrophage phenotype, while antibody 25F9 stains cells in the differentiation pathway from monocytes to macrophages. The total numbers of labeled cells were determined in connective tissue beneath sulcular epithelium, connective tissue beneath oral epithelium and middle connective tissue. The investigators reported a significantly higher incidence of specimens expressing 27E10 positive cells throughout the oral epithelium in CSA treated group when compared to control and nifidipine groups. In addition, the number of RM3/1-positive macrophages was significantly greater in the CSA group in connective tissue beneath oral epithelium than in the control group. Another study done by Pernu and Knuuttila (2001) was aimed at evaluating the differences in macrophage (CD68) and lymphocyte subpopulations (CD4, CD8 and CD20) in nifidipine and cyclosporine associated gingival overgrowth. To test this objective, biopsy samples of overgrown gingival tissues from patients receiving either of the two medications were taken and compared to samples taken from healthy gingiva. The authors divided the connective tissue area in each section into three different zones: 1- the connective tissue beneath the sulcular epithelium, 2- the middle connective tissue and 3- the connective tissue beneath the oral epithelium. They observed that in the nifidipine group, the proportion of CD8 labeled cells was significantly higher in the connective tissue beneath the sulcular epithelium when compared to control group. In both medicated groups the proportion of CD68 labeled cells was higher in all three zones than in control group. On the other hand, there were no significant differences between medicated and control groups regarding CD4 and CD20 labeled cells.

In another study, Iacopino *et al* (1997) observed that the inflammatory subset (27E10- the macrophage), which produces significantly greater amounts of IL-1β, is found in severely inflamed gingival tissue but not in drug-induced gingival overgrowth. This finding contrasts the study of Nurmenniemi *et al* (2002). The reparative/proliferative subset (RM3/1 macrophage) produces

significantly greater amounts of PDGF-B and is found in PHT-induced hyperplastic tissue and not in severely inflamed tissue. These data appear to support the hypothesis that macrophage phenotype may determine whether tissues enter into a state of breakdown, repair/maintenance, or proliferation.

-Fibroblasts:

In order to examine the cellular mechanisms pertinent to CSA-induced gingival overgrowth, Barber *et al* (1992) performed a study to observe the effect of CSA on fibroblasts. The fibroblasts that were used in this research were taken from 3 different sources: 1- normal healthy human gingiva, 2- cyclosporine-induced overgrown gingiva and 3- human fetal lung. In addition, they also analyzed the effect of *Escherichia coli* LPS on the same cells. The results demonstrated that cells taken from the gingival overgrowth, when exposed to different concentrations of CSA, were not stimulated to synthesize DNA at a higher level compared to controls. Cells from normal gingiva and fetal lung displayed an increased proliferative activity in response to CSA. It was concluded that CSA has the capacity to stimulate fibroblast proliferation directly. The fact that cells from overgrown gingival did not demonstrate a significant response to CSA support this finding that CSA-induced gingival overgrowth does not represent a cellular hyperplastic response. There was also a synergistic effect noted when the proteoglycan output of normal gingival cells was assessed in response to co-incubation with both CSA and LPS (Barber *et al* 1992). So, based on this latter finding, it might be concluded that bacterial LPS may be an important co-factor in the pathogenesis of CSA-induced gingival over growth. Cotrim *et al* (2003) performed a study to analyze the effect of CSA and TGF-β1 on the proliferation of the normal gingival (NG) fibroblasts. They observed that CSA at the concentrations of 50 -200 ng/ml increased NG fibroblasts proliferation, with the maximal stimulation observed at 200 ng/ml. With increasing concentration of CSA (400 & 800 ng/ml) NG fibroblasts proliferation was

inhibited. They also reported that the proliferating cell nuclear antigen (PCNA) levels in NG fibroblasts that were incubated with 200 ng/ml of CSA were also significantly higher than those of controls. In addition, the authors studied the effect of incubation with TGF-β1 for 24 hours on these cells and observed a significant increase in the proliferative rate of NG fibroblasts at a TGF-β1 concentration of 0.1-1 ng/ml. However, at a concentration of 10 ng/ml TGF-β1 decreased NG cell proliferation. Cortim and colleagues concluded that; 1) CSA stimulates NG fibroblast proliferation at the same concentrations found in the serum of patients undergoing CSA treatment (human serum level range: 100-250 ng/ml), and 2) CSA stimulates NG fibroblast proliferation through autocrine stimulation of TGF-β1.

-Mast cells:

Pisanty *et al* (1990) used both light microscopyand SEM to study gingival samples of patients suffering from Behcet's syndrome who were treated with CSA for up to 20 months. They demonstrated the presence of mast cells, both intact and degranulated, in the gingival epithelium. In another study, to observe the ultrastructural and histochemical aspects of cyclosporine-induced gingival overgrowth Mariani *et al* (1996) obtained tissue samples from kidney transplant patients. They observed a high numbers of mast cells in overgrown tissues. Based on this finding, they stated that mast cells presence could result in a high release of histamine, favoring the transfer of fluids from blood to the amorphous ground substance by means of the micropynocytotic vesicles of the capillaries. The authors concluded that the mast cells may contribute significantly to the accumulation of abnormally high quantities of intercellular liquid. On the other hand, Asahara *et al* (2000) used mast cell deficient mice to determine the role of mast cells in CSA-induced gingival overgrowth. They fed this group of mice and a control group with different concentrations of cyclosporine daily. After 30

days the mice were sacrificed to get the gingival sections. Both groups, fed with 600 mg/kg/day of cyclosporine, demonstrated gingival hyperplasia. They concluded that the mast cells are not necessary in the development of the overgrowth and that the increased number of mast cells in the enlarged gingiva observed by Mariani *et al* (1996) may be a secondary effect of gingival hyperplasia.

-Keratinocytes:

In order to compare the mitotic activities of keratinocytes in sulcular, oral sulcular, and oral gingival epithelium in nifedipine and CSA-induced gingival overgrowth tissue and in normal gingiva, Nurmenniemi *et al* (2001) obtained gingival samples from the above groups. Cryostat sections were stained with monoclonal antibody for Ki-67 and the mitotic activity of epithelial cells was determined as a percentage of the labeled cells in relation to total numbers of epithelial cells in the basal layers of 3 different zones. It was observed that mitotic activities were significantly higher in medicated groups when compared to controls. The difference was most clearly seen in the oral epithelium. This study concluded that the epithelial thickening in nifedipine- and CSA-induced gingival overgrowth is associated with increased mitotic activity. Differences in mitotic activity between the oral epithelium, oral sulcular and sulcular epithelium suggest that local gingival inflammation affects the drug-induced changes.

4-2 Growth Factors:

-Platelet-Derived Growth Factor (PDGF):

PDGF is a dimeric polypeptide, consisting of A and B chains, in homodimer (AA, BB) or hetrodimer (AB) combinations. The A chain is believed to play a minor role in cell proliferation and tissue repair. The B chain, however, is a potent mitogen for cells of mesenchymal origin and has been demonstrated

to promote growth and healing of connective tissues. Additionally, PDGF-B is a major chemoattractant for fibroblasts, stimulating fibroblast proliferation and synthesis of glycosaminoglycans, proteoglycans, fibronectin, and collagen. PDGF is released from platelets, macrophages, endothelial cells, smooth muscle cells and fibroblasts. In terms of proliferative activity, the release of PDGF-B from macrophages is believed to be responsible for connective tissue proliferation associated with inflammation. Thus, the increased levels of PDGF-B may promote fibroblast proliferation and/or fibroblast production of extracellular matrix constituents in hyperplastic tissues (Nares *et al* 1996).

In situ hybridization studies performed on tissues from control and CSA- treated patients exhibiting gingival overgrowth have shown that administration of CSA increases macrophage PDGF-B gene expression (Plemmons *et al* 1996).

Nares and colleagues (1996) performed a study to quantify PDGF-B gene expression in hyperplastic tissues in patients receiving CSA therapy. They extracted the total RNA from human gingival samples obtained from CSA-treated and control patients. In addition, dual fluorescence immunohistochemistry for mature macrophage marker antigen (CD51) and intracellular PDGF-B was performed. The study demonstrated a significant increase in PDGF-B mRNA in hyperplastic tissues from CSA-treated patients. Since CSA caused a 48-fold increase in PDGF-B mRNA compared to 8-fold increase in the amount of this growth factor caused by inflammation, the authors concluded that CSA-induced increase was inflammation-independent. PDGF-B producing cells were also identified as mature macrophages with a non-uniform distribution limited to the papillary connective tissue. They hypothesized that the macrophages may play a primary role in this process through CSA-stimulated up-regulation of various growth factors/cytokines. Similarly, in an animal study, Iacopino *et al* (1997) tested rat macrophages for PDGF-A/ PDGF-B synthesis in response to various levels of CSA, using an

ELISA assay. The results demonstrated that CSA produce a significant increase of PDGF-A/B levels in conditioned media in all concentrations.

-Connective tissue growth factor (CTGF):

CTGF was first identified as a cysteine-rich polypeptide secreted by vascular endothelial cells in culture. CTGF functions as both an autocrine and paracrine-signaling molecule to maintain and perhaps to amplify/synchronize the response of fibroblastic cell proliferation and extracellular matrix synthesis. Elevated levels of CTGF occur in skin sclerosis, renal fibrosis and lung fibrosis. The close association of CTGF with vascular endothelial cells led to the concept that CTGF could also play an important role in angiogenesis. It has been suggested that CTGF could facilitate the growth of fibroblasts in part by a mechanism involving increased angiogenesis during development and wound repair. CTGF is strongly and rapidly induced by TGF-β in vivo and in vitro (Uzel *et al* 2001). These authors investigated the presence and distribution of CTGF protein by immunohistochemistry in tissue samples taken from patients undergoing therapy with phenytoin, nifidipine and cyclosporine. The data from their study showed significantly higher CTGF staining in phenytoin-induced gingival overgrowth tissues compared to controls, CSA, or nifidipine-induced gingival overgrowth.

-Transforming growth factor-β (TGF-β):

In mammals there are three TGF-β isoforms; β1, β2 and β3.These can be distinguished by their effects on cell growth, cytokine expression and receptor binding characteristics. They are multifunctional cytokines known to be important in both healing and fibrosis The TGF-β isoforms can be expressed by most cells, including gingival inflammatory cells, endothelial cells and fibroblasts (Border &

Rouslahti 1992). Increased TGF-β expression may be associated with cyclosporine-induced renal fibrosis (Wondimu *et al* 1997).

James *et al* (1998) investigated the potential role for TGF-β1 in pathogenesis of CSA-induced gingival overgrowth. In their in-vitro study they determined that the cellular activity of gingival fibroblasts is dependent on culture conditions and that fibroblasts derived from overgrown gingival tissue are more responsive to TGF-β1 than normal gingival fibroblasts when cultured in type I collagen gel.

Coletta *et al* (1999) believed that TGF-β1 is a key mediator of tissue fibrosis causing extracellular matrix accumulation in pathologic states such as hereditary gingival fibromatosis.

Wright *et al* (2001) performed a study to determine whether there is any altered expression of TGF-β isoforms or their receptors in tissue from patients with drug induced (PHT, CSA, NIF) gingival overgrowth or hereditary gingival fibromatosis compared to the controls. They observed that fibroblasts from both drug-induced gingival overgrowth and hereditary gingival fibromatosis expressed more TGF-β1 compared to controls. Cells expressing TGF-β2 were present at control levels in DIG but were significantly reduced in hereditary gingival fibromatosis. In contrast the number of TGF-β3 positive cells was the same in overgrowth tissues and controls. They concluded that qualitative differences in TGF-β isoforms and receptor expression by fibroblasts in gingival overgrowth may contribute to disease pathogenesis. However, one critique of this study is that they had both healthy periodontium and periodontitis samples in their control group. Since we expect to see alterations in growth factors in association with periodontitis, this group is not the most suitable one to use for comparison.

In the study of Saito *et al* 1996, it was noted that there were increased numbers of fibroblast-like cells that were positive for TGF-β1 in the lamina propria of nifidipine and phenytoin-induced gingival overgrowth tissues compared to control gingiva.

Budneli *et al* (2001) investigated the level of TGF-β1in gingival crevicular fluid (GCF) samples of CSA-treated patients and compared the results with control groups. All the patients in CSA group exhibited severe gingival overgrowth. Control groups included both patients with gingivitis and subjects with healthy periodontium. GCF samples were harvested from sites exhibiting gingival overgrowth (GO+) and sites not exhibiting gingival overgrowth (GO-). The TGF-β level was analyzed by ELISA. The results demonstrated that the concentration of TGF-β1 in sites with and without gingival overgrowth were similar and significantly higher than that of healthy sites. They also showed an increased amount of TGF-β1in gingival overgrowth group compared to gingivitis group, although the differences were non-significant. The authors supported the theory that CSA increases the synthesis of TGF-β1 in GCF. However, they concluded that due to lack of statistically significant differences between CSA GO+ and CSA GO-, it seems unlikely that GCF TGF-β1 is the sole factor responsible for the gingival overgrowth. The authors did not mention whether the patients in CSA group or even control patients were taking any other type of medications.

In an in-vitro study (Cortim *et al* 2002) fibroblasts from normal human gingiva were admixed with different concentrations of CSA (0, 100 or 200 ng/ml of CSA) and cultured for 24 hours. Using reverse transcriptase-polymerase chain reaction (RT-PCR), the expression and production of TGF-β1 was then determined. MMP and TIMP expression levels were also analyzed by the same technique. In addition, in order to determine the effect of TGF-β1 on the expression of MMP and TIMP by human gingival fibroblasts incubated with CSA, the cells were treated with sense oligonucleotides (SON) or antisense oligonucleotides (AON). The result of this study demonstrated that CSA stimulates

expression and production of TGF-β1, while it inhibits expression of MMP-1 and MMP-2 by gingival fibroblasts. TIMP expression was not statistically significant. Furthermore, the authors observed that blocking TGF-β1 synthesis with AON resulted in an increased effect of CSA on the MMP-1 and MMP-2 expression by gingival fibroblasts, whereas TIMP-1 and TIMP-2 were not affected. In conclusion, the authors stated that TGF-β1, acting in an autocrine fashion, may contribute to a reduction of proteolytic activity of human gingival fibroblasts in CSA-induced gingival overgrowth, which favors the accumulation of extracellular matrix.

-Basic fibroblast growth factor (bFGF):

Saito *et al* (1996) demonstrated that the increased synthesis of bFGF as well as TGF-β and their respective receptors may be related to the pathogenesis of drug-induced gingival over growth. Hong and Trackman (2002) performed a study to determine whether cytokines reported to be present at elevated levels in drug-induced gingival overgrowth regulate lysyl oxidase, elastin or α-1 type I collagen when administered to human gingival fibroblasts. They obtained samples from systemically healthy individuals. They treated the fibroblasts with different concentrations of IL-1β, bFGF and PDGF-BB. The results demonstrated that bFGF significantly reduced fibroblast lysyl oxidase and α-1 type I collagen mRNA levels in a dose- and time-dependent manner. Controversially, no significant changes were observed using IL-1β and PDGF-BB. The authors concluded that the strong downregulation of lysyl oxidase and collagen type I by bFGF suggested the role for this cytokine might be regulatory. As such it could limit stimulation of connective tissue synthesis induced by other fibrogenic factors such as TGF-β1 and CTGF that are known to be present in gingival tissues. Alternatively, as bFGF is mitogenic and angiogenic, a possible role for bFGF may be to stimulate cell proliferation and neovascularization that could ultimately contribute to gingival overgrowth.

-Keratinocyte growth factor:

Keratinocyte growth factor stimulates the growth and activity of epithelial cells via the keratinocyte growth factor receptor (Das *et al* 2002).

4-3 Cytokines:

It has been postulated that CSA-induced alterations of cytokine levels in gingival tissues might play a role in the drug-induced gingival overgrowth.

-Interluekin-1 (IL-1):

IL-1 is the term for two polypeptides, IL-1α and IL-1β. They possess a wide spectrum of inflammatory, metabolic, physiologic, and immunologic properties. Although both forms of IL-1 are produced by different genes, they recognize the same cell surface receptors and share the similar biologic activities. IL-1β belongs to a group of cytokines (pro-inflammatory cytokines) with overlapping biological properties. IL-1β is produced predominantly by macrophages, but it can also be released from platelets, fibroblasts, endothelial cells, and keratinocytes. In macrophages, significant amount of IL-1β are not observed in health. However, there is a dramatic increase in IL-1β in response to infection, microbial toxins, inflammatory agents, products of activated lymphocytes and complement (Jobling *et* al 1988- Iacopino *et al* 1997). Many studies have demonstrated that the concentration of IL-1β appears to be a major determinant in the progression and extent of tissue degradation in chronic inflammation (Stashenko *et al* 1991, Heasman *et al* 1993). IL-β has also been demonstrated at increased levels in inflamed gingival tissue and gingival crevicular fluid harvested from periodontally diseased sites. It is also among the most potent inducers of bone resorption (Howells 1995). In terms of extracellular matrix turnover IL-1β induces connective tissue degradation

by inducing increased expression of the matrix metalloproteinases collagenase and stromelysin, while reducing levels of tissue inhibitor of metalloproteinases (TIMP) (Birkedal 1993).

To investigate the effects of CSA on the production of some cytokines, including IL-1β, Myrillas *et al* (1999) obtained human gingival fibroblasts from biopsies of normal gingival tissue from healthy volunteers and from biopsies of patients with cyclosporine induced gingival overgrowth. The results of this study demonstrated that CSA induced a statistically significant inhibition of IL-1β production by peripheral mononuclear cells.

In the study of Iacopino *et al* (1997), no increase in amount of IL-1β was found when macrophages were treated with CSA, in contrast to the increase in the level of PDGF-B as noted above.

Several studies have shown that there is a positive correlation between the degree of inflammation and gingival crevicular fluid (GCF) cytokine levels (Masada *et al* 1990). In order to determine the levels of IL-1β in GCF, Atilla *et al* (1998) collected GCF samples from renal transplant patients receiving CSA with and without gingival overgrowth and compared them to samples taken from unmedicated individuals with gingivitis as well as to samples from subjects with a clinically healthy periodontium. They concluded that CSA therapy does not alter GCF IL-1β levels, while gingival inflammation plays a significant role in the elevation of GCF IL-1β levels.

-Interleukin-6 (IL-6):

IL-6 is produced by a variety of cells, including T-cells, B-cells, monocytes, keratinocytes, endothelial cells, some tumor cells and fibroblasts. While IL-6 produces many signals for these cells, one proposed action is to inhibit fibroblast growth (Kishimoto 1989). In terms of connective tissue turnover, IL-1β and IL-6 have opposing effects with IL-1β inducing tissue breakdown through the induction of MMPs secretion and IL-6 reducing tissue breakdown through increased TIMP expression.

In addition, IL-6 has been shown to stimulate the proliferation of human gingival keratinocytes, an effect which could account for the epithelial hyperplasia observed in overgrown gingival tissues (Birkedal-Hansen 1993). In the study of Myrillas *et al* (1999), the production of IL-6 by gingival fibroblasts and peripheral blood mononuclear cells (PBMC) under the effect of CSA was investigated. Results revealed that at a concentration of 2000 ng/ml, CSA stimulated IL-6 production by PBMC. They also observed that fibroblasts derived from overgrown gingiva produced significantly higher levels of IL-6 than their normal counterparts. The authors concluded that CSA-induced gingival overgrowth tissue contained significantly higher levels of IL-6 than inflamed or normal gingiva. However, the results of this study contrast those of a study done by Yoshimura *et al* (1991), which demonstrated that CSA significantly reduced serum levels of IL-6. The study was performed on serum and IL-6 production of human peripheral blood mononuclear cells.

Williamson *et al* (1994) also performed a study to determine whether CSA therapy affects IL-6 gene expression in gingival tissues. They obtained samples from individuals who received kidney or liver transplant and were on CSA therapy and compared those to samples taken from healthy individuals. In all test subjects, gingival overgrowth was determined to be moderate. Radioimmunoassay and in-situ hybridization demonstrated significantly elevated IL-6 levels in CSA-treated tissues. The authors concluded that CSA causes an upregulation of IL-6 gene expression in human gingiva. The finding suggests a mechanism for inducing gingival overgrowth. Studying GCF samples of patients receiving CSA with and without gingival overgrowth, Atilla *et al* (1999) stated that CSA therapy does not alter GCF IL-6 levels directly, and that inflammation probably plays an important role in the elevation of these cytokines.

4-4- Integrins:

Integrins are the principle mediators of the molecular dialogue between a cell and its extracellular matrix environment. The unique combinations of integrins subunits determine which extracellular matrix molecules will be recognized by a cell (Gumbiner *et al* 1996). Both $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins are cell surface receptors for type I collagen in fibroblasts. Based on the study of Lee *et al* (1996), the initial binding step of collagen phagocytosis relies on adhesive interaction between fibroblasts and collagen, and that $\alpha 2$ integrin plays a critical role in the phagocytic regulation of collagen internalization. Kataoka *et al* (2003) studied the expression of $\alpha 2$ integrin in fibroblasts derived from gingiva of rats fed a diet with or without CSA, and investigated whether the difference in expression of $\alpha 2$ integrin is involved in the regulation of collagen phagocytosis. The findings of their study revealed that fibroblasts from CSA-treated gingiva showed less phagocytosis. It was also confirmed that CSA inhibited the expression of $\alpha 2$ integrin in fibroblasts. The level of $\alpha 2$ integrin mRNA in the fibroblasts isolated from CSA treated rat gingiva was apparently lower than that of the control fibroblasts. Further studies were suggested to clarify the role of $\alpha 2$ integrin in the induction of CSA induced GO.

4-5-Matrix Metalloproteinases (MMPs):

The matrix metalloproteinase (MMP) family consists of at least 13 members with closely related domain structures and discrete functions. All have a 21-kd catalytic domain that contains a Zn++ binding site. MMP family is responsible for the remodeling of extracellular matrix in both physiological and pathological conditions. MMP-1 or collagenase is produced by a variety of cell types including keratinocytes, fibroblasts, and macrophages. This enzyme is an important regulator of connective tissue remodeling and is present in high concentration in inflamed gingival regions,

including those affected with periodontal disease; whereas in hyperplastic tissue and fibrosis we expect to find a lower level. MMP-1 can hydrolyze type I, II, III, VI, VIII and X collagens. The gelatinase group of MMPs has 2 prominent members, MMP-2 and MMP-9. Both of these enzymes have a high affinity for gelatin. Stromelysins have a broad specificity with the ability to degrade proteoglycans, basement membrane, laminin, fibronectin, in addition to collagens. Three stromelysins have been described so far; stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and stromelysin-3 (MMP-11) (Bartold & Narayanan 1998, Tuter *et al* 2002). MMPs are all specifically inhibited by tissue inhibitor of metalloproteinase-1 (TIMP-1). TIMPs are expressed by many cells including fibroblasts, keratinocytes, monocytes/macrophages, endothelial cells and osteoblasts (Meikle *et al* 1994).

Balzani *et al* (2000) investigated the production and activity of MMPs in CSA-induced gingival overgrowth. They utilized both animal models and in vitro cell cultures to study this issue. Thirty six male rats received injections of CSA daily for 60 days. For the in vitro study gingival fibroblasts, obtained from seven healthy volunteers were incubated with cyclosporine. The results demonstrated that the gingival overgrowth tissue had lower gelatinolytic activities of MMP-2 than the control gingival tissues in rat. In addition it was observed that CSA significantly inhibits, at the protein level, the production of both MMP-1 and MMP-3 by gingival fibroblasts. The authors noted that inhibition of MMPs occured at CSA concentration near those found in the serum of CSA immunosuppressed patients. They concluded that there is a co-operative role of MMP-1 and MMP-3 in the pathogenesis of CSA-induced gingival overgrowth (inhibition of these MMPs may contribute to an abnormal accumulation of fibronectin and proteoglycans). In another study done by Yamada *et al* (2000) normal human gingival fibroblasts were cultured with or without phenytoin or cyclosporine A and the total RNA and cellular proteins were collected daily for RT-PCR analyses and measurement of lysosomal

enzyme activity. The results revealed that these medications suppressed the expression of MMP-1 and TIMP-1 and cathepsin L, but not the cathepsin B in a time-dependent manner. They concluded that the decreased ability of protein degradation by lysosomal enzymes is, at least, one of the factors in the pathogenesis. In another study, Cotrim *et al* (2002) demonstrated that CSA inhibited expression of MMP-1 and MMP-2 by human gingival fibroblasts taken from normal tissue, whereas it has little effect on TIMP-1 and TIMP-2 expression. Tuter *et al* in 2002 reported conflicting findings when comparing MMP-1 and TIMP-1 levels in cell cultures of gingival tissue derived from both renal transplant patients receiving CSA and exhibiting gingival overgrowth and periodontally healthy control subjects. They also investigated the effects of CSA on MMP-1 and TIMP-1 levels in CSA-treated gingival fibroblast cultures derived from periodontally healthy subjects. They classified gingival fibroblast cultures as: 1- CSA GO (gingival overgrowth) 2- H+CSA (CSA treated healthy gingival fibroblast culture) and 3- H (healthy gingival fibroblasts). The results showed the levels of TIMP-1 were significantly lower in CSA GO than H. There was no statistically significant difference in the levels of MMP-1 between H and CSA GO. The authors concluded that CSA therapy does not have a significant effect on MMP-1 levels; however, low TIMP-1 levels can be an important factor in the pathogenesis of CSA GO. Silva *et al* (2001) used a rat molar extraction model to observe the influence of CSA-induced immunosuppression on MMPs activity in dental socket granulation tissue. In their study it was observed that CSA inhibits the activity of MMP-2 and MMP-9 in the early phase of granulation tissue in the healing dental sockets. However, some enhanced activities of MMP-1 was observed, which is in agreement with the study of Tuter *et al* (2002).

4-6-Proteoglycans and Glycosaminoglycans:

Proteoglycan is a family of macromolecules composed of one or more of glycosaminoglycans (GAGs) covalently bound to a protein core (Balazs 1967, Roberts 2003). GAGs are the principle carbohydrate components of proteoglycans and they include hyaluronan, chondroitin-4-sulfate, dermatan sulfate, heparin sulfate, heparin and keratan sulfate. Except keratan sulfate, all of the GAGs are composed of repeating disaccharide units of uronic acid and hexosamine. Keratin sulfate contains D-galactose in the place of uronic acid. In addition to the glycosaminoglycan chains, smaller oligosaccharides have been identified in most proteoglycans (Bartold & Narayanan 1998). Proteoglycans are large, highly anionic glycoproteins of connective tissues, which are located within the matrix as integral components of the matrix structure as well as on cell surfaces and within cell organelles. Their functions include tissue hydration, regulation of collagen fiber formation, growth factor binding, cell adhesion, and cell growth. Proteoglycans are classified into at least three separate groups; matrix organizers and space fillers, cell surface proteoglycans, and intracellular proteoglycans of the hematopoietic cells (Bartold & Narayanan 1998). Versican, an important member of the first group, was originally isolated from fibroblasts. Since then it has been found to be present in a wide variety of tissues including aorta, brain, cartilage, placenta as well as skin (Ruoslati 1989). The ultrastructural study of Mariani *et al* (1993) demonstrated a particular abundance of amorphous ground substance in connective tissue of CSA-induced gingival overgrowth. Bensada *et al* (1995, 1997) have demonstrated that versican is consistently associated with proliferating myofibroblasts in early lesions of pulmonary fibrosis, but absent from areas of dense collagen deposits consistent with a transient role for versican in the cell biology of proliferative lung disease and its degradation concomitant with myofibroblasts apoptosis.

5- Histological features of CSA- induced gingival overgrowth:

5-1-Epithelium: In a histologic study of CSA induced gingival overgrowth Miriani *et al* (1996) found that the basal and spinous layers of epithelium show distinct dilatation of the intercellular spaces. They found the dimensional increase in gingival overgrowth to be due to an increased production of amorphous ground substance by fibroblasts. Other histologic features being described for CSA-induced gingival overgrowth are epithelial ridges penetrating deep into CT and highly vascularized CT with focal accumulation of infiltrating inflammatory cells. The predominant inflammatory cell type is reported to be the plasma cell, with lymphocytes seen to a lesser degree. Acanthosis and parakeratinization of the epithelium with psuedoepithelioumatous proliferation can be observed. Immunohistochemical examination of CSA induced gingival overgrowth exhibits active protein synthesis and reduced cytotoxic or degenerative changes.

5-2-Connective Tissue:

According to Rateitschak *et al* (1983), the connective tissue of CSA-induced gingival overgrowth exhibited irregularly arranged collagen bundles and high vascularization. Focal accumulations of infiltrating inflammatory cells were also observed. Mariani *et al* (1993) also report the presence of amorphous ground substance in the connective tissue. According to the authors, this substance passes through the spaces between adjacent basal cells, which at some points assumed the features of intercellular canals.

6- Prevention, Treatment and Maintenance of gingival overgrowth:

The general consensus is that a rigorous oral hygiene program is extremely important in the preventive and therapeutic management of drug induced gingival overgrowth and should be instituted prior to

starting such therapy whenever possible (Hallmon & Rossmann 1999). Philstrom *et al* (1980) studied the effectiveness of a preventive dental program in 13 patients who were taking phenytoin (PHT). All patients received OHI and SRP within 30 days of initiating PHT therapy. Recall appointments were repeated every 3 months. They observed a small increase in gingival enlargement anteriorly during the first 6 months with no further progression.

Fu *et al* (1997) examined the role of plaque retention on CSA induced gingival overgrowth in rats. Ligatures were placed around the first molar unilaterally and the rats administered CSA in different dosages. Gingival overgrowth was significantly increased in sites with higher CSA dosage and longer treatment duration. Gingival overgrowth was enhanced in ligated sites regardless of CSA dose. They suggested that plaque retention magnifies CSA-induced gingival overgrowth. Ingeli *et al* (1999) studied 38 patients who were on CSA or NIF. All patients received initial periodontal therapy, after which anterior segments in each patient were surgically treated. Surgical therapy consisted of the flap technique with a 90 degree gingivectomy incision. Patients were placed on a maintenance therapy for 18 months. Recurrence was observed in 34%. Age, gingival inflammation, and attendance at recall appointments were significant determinants for the recurrence of severe gingival overgrowth. Pilatti and Sampaio (1997) histologically assessed the influence of daily applied 0.12% CHX on the severity of CSA-induced gingival overgrowth in rats. They concluded that the use of CHX may lead to an improved plaque and gingivitis control, which may help reduce the severity of gingival overgrowth. Camagro *et al* (2001) in their article mentioned that consideration should be given to the possibility of changing medications in consultation with the patient's physician. Alternative medications to phenytoin include carbamazepine and valproic acid, both of which have been reported to have a lesser impact in inducing gingival overgrowth. Diltiazem and verapamil are other calcium channel blockers exhibiting a lesser prevalence of gingival enlargement (20% and 4% respectively in compare to 44%

reported for NIF). It also has been shown that CSA induced gingival enlargement can spontaneously resolve if the drug is substituted by tracolimus. There is also preliminary evidence that the antibiotic azithromycin may aid in decreasing the severity of CSA induced gingival overgrowth. They recommended that whenever the drug substitution is attempted, it is important to allow for 6-12 months to elapse between discontinuation of the offending drug and the possible resolution of gingival enlargement. When gingival enlargement persists, despite drug substitution and good plaque control, these cases need to receive surgical treatment, which could be either gingivectomy or periodontal flap therapy. The advantages of gingivectomy are simplicity and quickness, but unlike the periodontal flap therapy, it will not allow for osseous recontouring and may sacrifice keratinized tissue. In addition, gingivectomy results in healing by secondary intention, which causes discomfort and an increased chance of post-operative bleeding. In general, small areas (up to six teeth) presenting with drug-induced gingival enlargement where there is no evidence of attachment loss can be effectively treated with gingivectomy. However, the amount of keratinized tissue should be considered in these cases. It is recommended that at least 3 mm of keratinized tissue in the apicocoronal direction remain after the surgery is concluded. Any situation in which the gingivectomy technique may result in the elimination of all keratinized tissue and consequent creation of mucogingival problems should be treated with the periodontal flap surgery (Camarego *et al* 2001).

Recurrence of drug-induced gingival enlargement is common in surgically treated cases (Rees *et al* 1995). Proper oral hygiene care, chlorhexidine rinse, and regular scaling and root planning can decrease the rate and the degree of recurrence (Saravia *et al* 1990). Recurrence may occur as early as 3-6 months after the surgical treatment, but in general, surgical results maintained for at least 12 months (Pilloni *et al* 1998).

Chapter 2- The objectives of the study:

The aim of this study is to characterize the tissue changes that occur in gingival overgrowth associated with cyclosporine A therapy in kidney transplant patients. In order to do this, cell and matrix populations in histological sections of gingival overgrowth biopsies will be compared with those of systemically healthy controls, which are obtained in the procedure of crown lengthening.

The objective of this study:

- Identify and select matched disease and control patients
- Systematically sample and process tissues to allow quantitative and semi-quantitative histological analysis of tissue changes associated with gingival overgrowth
- Conduct and analysis of tissue changes in the epithelium and connective tissue of the gingival overgrowth samples and compare it to the ones in the control group

The hypothesis is that immunosuppressive drugs may have a direct effect on growth factor and cytokine synthesis and release in the oral cavity resulting in a proteoglycan-myofibroblast complex that is responsible for gingival overgrowth. The nature of the matrix molecules and cell populations involved will be determined.

Chapter 3- Materials and Methods:

The collection of clinical information and tissue samples took part in Shahid Beheshti (SHB) School of Dentistry, private practice, and the kidney transplant center of Labafi hospital in Tehran, Iran. It included patient selection, oral examination, periodontal surgery and collection of tissue samples at the time of surgery. This study was approved by the research committee of SHB School of Dentistry.

The procedure and reasons for the study were explained to the patients in both groups and patients all gave their informed consent to use of surgical samples for research. For those patients under the age of eighteen, the consent forms were signed by one of the parents. (Appendix 1 shows the consent form both in Persian and English).

The study aimed to compare the following two groups of patients: 1- Gingival overgrowth group and 2- Control group.

1- Gingival overgrowth group (The GO group):

The gingival overgrowth group consisted of 18 individuals (6 females and 12 males with an age range of 16-50 years) who had all undergone kidney transplant surgery at least one year prior to this study and were taking cyclosporine-A. All of these patients demonstrated gingival overgrowth of degree II or III based on McGaw index (McGaw *et al* 1988). According to this index, the gingival overgrowth is classified as follows:

- Grade 0: No overgrowth or feather-edged gingival margin.
- Grade I: Blunting of gingival margin.
- Grade II: Moderate gingival overgrowth, < 1/3 of crown length.
- Grade III: Gingival overgrowth, >1/3 of crown length.

These patients were selected from those referred to Labafi hospital in Tehran, Iran for their regular blood pressure and renal function post-transplant check-up. This selection was conducted by a Periodontics resident and a trained dental student. The steps for patient selection were as follows:

- Reviewing of charts of patients who had an appointment on the same day.
- Initial oral examination on those who had had kidney transplant for at least one year and were prescribed CSA.
- Selection of individuals with Grade II or III GO from the above group and explanation of the study to them; informed consent.
- Clinical examinations and treatment.

2- Control Group:

Control group consisted of 12 systemically healthy individuals, sex and age-matched with the GO group (4 females and 8 males with an age range of 24-56 years). These individuals were referred to the Periodontics department of SHB dental school to receive a crown lengthening procedure for restorative purposes. Their clinical diagnosis was gingivitis and, based on the information obtained from their charts, they did not appear to have periodontitis. The purpose of this study was explained to them and they were asked to read and sign the consent form to give us permission to use the excised tissue for research purposes. Tissue excision is generally a part of the crown lengthening procedure. This tissue was saved instead of being discarded as would normally be the case.

3- Clinical Examinations:

General information including demographic data, general health concerns other than kidney problems, medications taken, and history of smoking was recorded for all patients in both groups. In the GO group, details regarding their kidney transplant were also collected based on information provided by the patients and/or from their medical charts in Labafi Hospital. These data included the reason for kidney failure, the date of transplant, the dosage of CSA and other medications taken, and the patients' serum levels of CSA. In the oral examination of this group Gingival Index (GI, Loe & Silness 1964), oral hygiene effectiveness, probing depth (PD) and gingival overgrowth index were recorded prior to any treatment. Consults with physicians were done for the gingival overgrowth patients regarding their systemic health and in order to know whether they required any antibiotic prophylaxis or a change in the amount of corticosteroid taken.

(Appendix 2 shows the form that was used to collect the clinical information).

4- Surgical Treatment:

Prior to surgical treatment, oral hygiene instruction and scaling/root planning were performed in both groups. All gingival overgrowth patients received periodontal flap surgery for accessing the bone surface and performing osseous corrections if required. Crown lengthening patients also received periodontal flap surgery. Following reverse bevel incision, the excised tissues were carefully removed and saved for the next step. After completion of the surgery, periodontal dressing (CoePak) was used in both groups and patients received post-op instructions. Chlorhexidine 0.12% (1 minute of rinsing, bid) was also prescribed. During the following visit, one week later, the periodontal dressing and sutures were removed and the patients were taught how to perform their oral hygiene. It was also explained to the GO patients that there is a possibility of recurrence of overgrowth, and in order to prevent this, they needed to be meticulous with their daily oral hygiene. Further, they were advised to

receive scaling/root planning (SRP) on a regular basis. Photographs were taken prior to and following surgery in the GO group. Periapical radiographs were also obtained to study the possibility of bone loss and also to ensure the absence of any pathologic condition in the surgical site. Surgeries in the GO and control groups occurred in private practice and at the dental school, respectively.

5- Tissue Collection:

Tissue samples were harvested from interdental papillae as well as marginal gingiva in both groups and they were immediately rinsed with normal saline and soaked in formalin 10% for 24 hours. That same day, the excised tissues were transferred to the pathology laboratory at the SHB dental school. The next day, the tissues were embedded in paraffin by the technicians of the pathology lab. At this point, they were safe and ready to be carried to Vancouver. Paraffin processing was done as follows:

1.	70% alcohol	45 minutes.
2.	70% alcohol	45 minutes.
3.	95% alcohol	45 minutes.
4.	95% alcohol	45 minutes.
5.	100% alcohol	45 minutes.
6.	100% alcohol	45 minutes.
7.	100% alcohol	45 minutes.
8.	Xylene	45 minutes.
9.	Xylene	45 minutes.
10,	Xylene	45 minutes.
11.	Paraffin wax	1 hour
12.	Paraffin wax	1 hour

6- Histochemical Staining:

Tissue sectioning and histochemical stainings were done in Department of Pathology at the UBC hospital. Initially, samples were cut to sections of 3 microns thickness.

De-paraffinizing was then performed using the following technique:

1. Xylene #1 for 3 minutes.
2. Xylene #2 for 3 minutes.
3. Xylene #3 for 3 minutes.
4. 100% alcohol #1 for 20 dips
5. 100% alcohol #2 for 20 dips
6. 100% alcohol #3 for 20 dips
7. 95% alcohol for 20 dips
8. Rinse slides in tap water for 1 minute.
9. Drain well before staining.

Three different techniques of staining were done including hematoxylin and eosin (H&E), Gomori's Trichrome-aldehyde fuschin stain (Gomori), and alcian blue pH 2.5- picrosirus red (AB/PSR). H&E staining was done to study general features of both epithelium and connective tissue, including changes in retepegs number, length and thickness, and inflammatory changes in connective tissue. Gomori staining was used to visualize collagen fibers, elastin and glycosaminoglycans in connective tissue. And finally, AB/PSR could detect the presence of proteoglycans and glycosaminoglycans (GAG) in connective tissue (blue) and collagen fibers (red).

7- Immunohistochemical Staining:

Immunohistochemical staining was performed in Dr. Roberts' laboratory. Sections were de-waxed in Xylene and brought through a graded series of alcohol to water, using the method described above. Detailed procedures were developed as needed for different antibodies. For staining, Vector system and Vectastain ABC kits were utilized. The technique is described in below:

1- Rinsing for 5 minutes in tap water.
2- Incubating the sections with 0.3% H_2O_2 for 30 minutes
3- Washing slides in Tris buffered saline (TBS) for 5 minutes
4- Incubating sections for 20 minutes with diluted normal blocking serum.
5- Blotting the excess serum from sections.
6- Incubating sections for 30 minutes with primary antiserum, no primary antiserum, or control antiserum diluted in TBS with 3% bovine serum albumin (TBS-BSA).
7- Washing slides in buffer for 5 minutes.
8- Incubating sections for 30 minutes with secondary antibody solution or no secondary antibody as control.
9- Washing slides in buffer for 5 minutes.
10- Incubating sections for 30 minutes with Vectastain ABC reagent.
11- Washing slides in buffer for 5 minutes.
12- Incubating sections in peroxide substrate solution to get the desired stain intensity (between 7-10 minutes).
13- Rinsing sections in tap water.
14- Counterstaining, clearing and mounting.

8- Antibody Staining:

Primary antibodies used in this study and their concentrations are collected in table 1.

Table 1: Primary antibodies, the concentrations and their targets.

Primary Antibody	Concentration	Target
1A4	1/1000	α-smooth muscle actin
PCNA	1/100	Proliferating cells
LC2	1/500, 1/1000, 1/5000	C terminal of Versican
2B1	1/200	Core of Versican
CD44	1/200	Hyaluronan receptors (HA) (Predominantly on macrophages)

9- Considerations to allow semi-quantitative analysis of immunohistochemical staining

The intensity of immunohistochemical (IHC) staining is influenced by many factors including:

- Concentration of antibody
- Time and temperature of incubation
- Section thickness
- Time and temperature of color development

In order to allow semi-quantitative comparison of tissue staining between the control and GO groups, we took the following precautions:

- Standardized tissue processing up to paraffin embedding
- Tissues to be compared were stored, sectioned and processed together.
- Sections to be compared were stained together in the same staining procedure.

10- Morphometric Analysis:

After reviewing the stained slides, we developed a number of histological grading schemes, to allow semi-quantitative analysis of histochemical and immunohistochemical data. In histochemical features, epithelial and connective tissue changes were studied using the following criterion:

10-1 Epithelial changes: Epithelial changes including presence or absence of acanthosis, hyperkeratosis, and parakeratosis as well as length and thickness changes of retepegs were recorded.

10-2 Inflammation: To assess the inflammatory infiltration, a rating scheme for the degree of inflammation was developed. Examples of each category are shown in the next page (see Figure1):

- Degree 0- Minimal infiltration
- Degree 1- Mild infiltration
- Degree 2- Moderate infiltration
- Degree 3- Severe infiltration

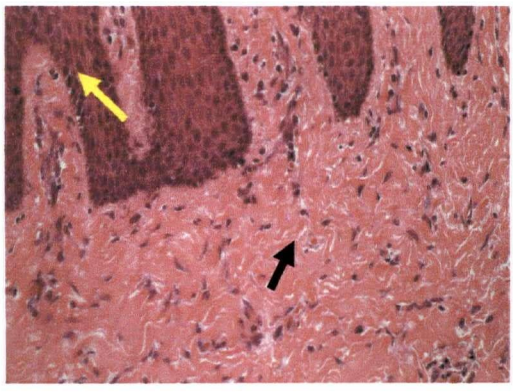

Fig 1-A

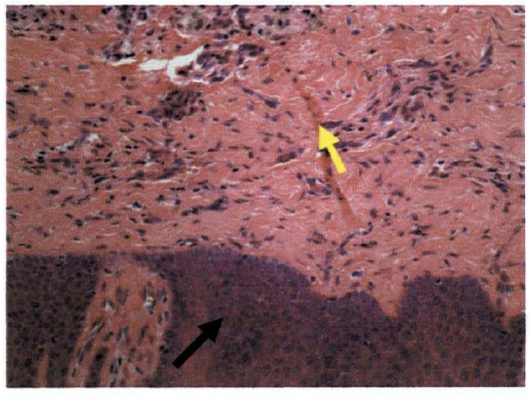

Fig 1-B

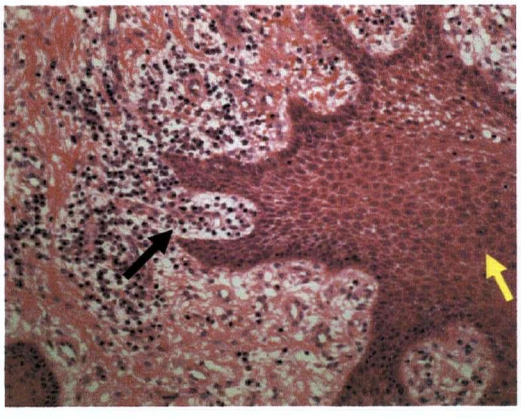

Fig 1-C

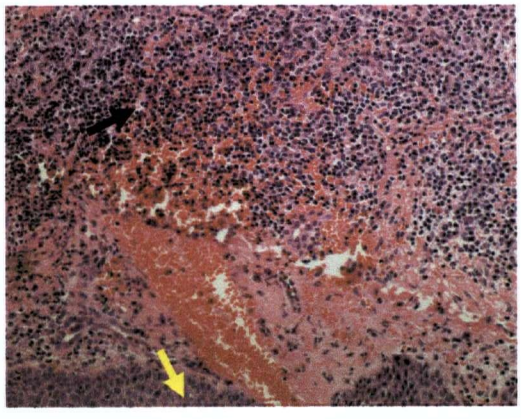

Fig 1-D

Figure 1- Different degrees of inflammatory infiltration in tissue samples.
A- Minimal infiltration of inflammatory cells, B- Mild infiltration of inflammatory cells, C- Moderate infiltration of inflammatory cells, D- Severe infiltration of inflammatory cells. Black arrow shows the inflammatory infiltration and yellow arrow points the epithelium. (original magnifications of all slides x 100)

Degree of inflammation in histological sections was assessed and recorded with reference to the scheme developed and standard images.

10-3 Connective tissue changes: Connective tissue changes including GAG accumulation and the amount of collagen and elastin fibers were assessed. In the histological features stained with AB/PSR, the blue staining areas were representative of GAGs accumulation. As for the inflammation scheme, a semi-quantitative grading scheme was developed as presented in the next page:

- Degree 0= Minimal amount of GAG accumulation
- Degree 1= Moderate amount of GAG accumulation
- Degree 2= Severe amount of GAG accumulation

The tissue sections were assessed for GAG accumulation according to this scheme. We also can observe the collagen bundles as red areas in these slides. Examples of different degrees are shown in Figure 2.

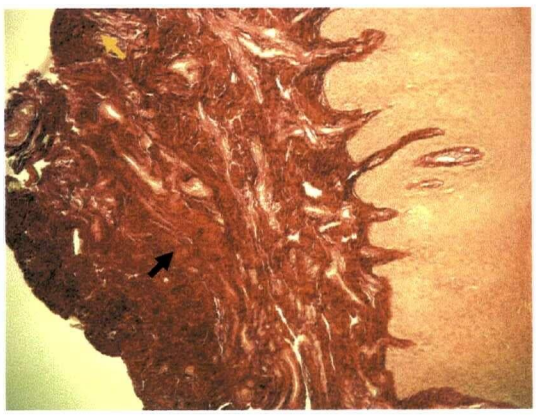

Fig 2-A

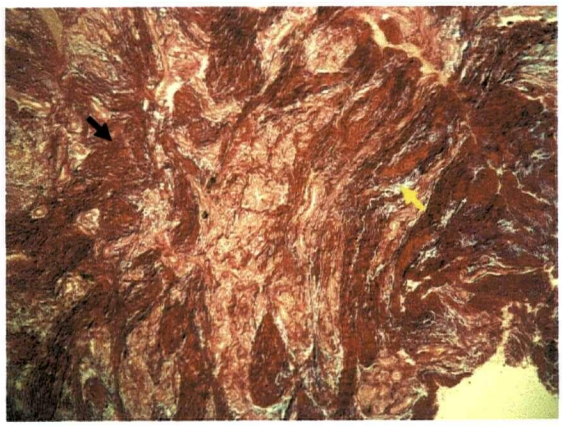

Fig 2-B

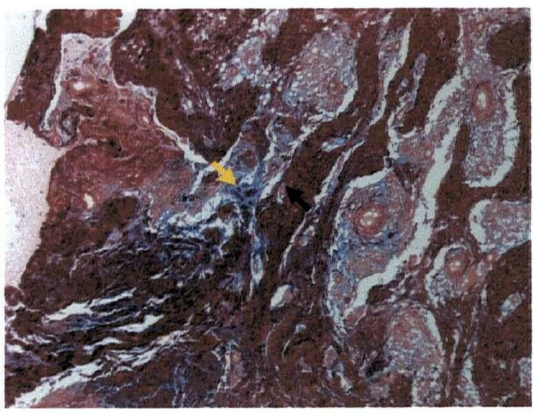

Fig 2- C

Figure 2. Different degrees of Alcian blue/ Picrosirius red staining in tissue samples.
A- Minimal or loss of GAGs accumulation and dense collagen bundles, B- Moderate accumulation of GAGs, C- Severe accumulation of GAGs. B and C both demonstrate the hydrated collagen. Yellow and black arrows show GAG accumulation and collagen bundles, respectively (original magnification of all slides x 100)

In Gomori's trichrome stained slides, which show the accumulation of collagen bundles, the criterion was based on presence or absence of hydrated or dense collagen (Figure3).

Dense and hydrated collagen could also be visualized in Alcian blue/PSR stained slides. In Gomori slides elastin fibers could also be detected. They were visualized as thin greenish fibers between the bundles of collagen.

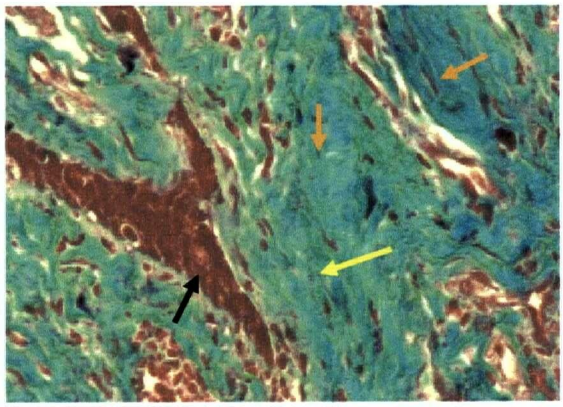

Fig 3-A

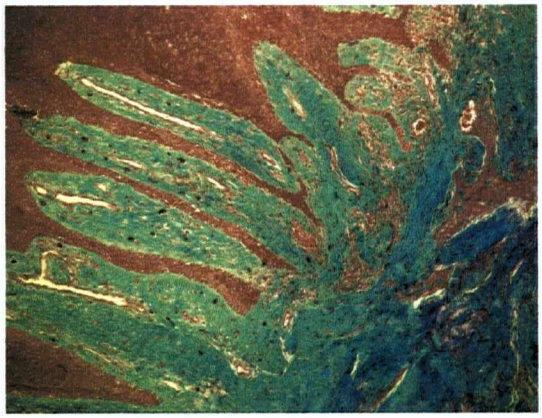

Fig 3-B

Figure 3- Gomori's trichrome staining- A- (magnification x 20) Gomori staining in gingival overgrowth group showing collagen bundles (Yellow arrow), retepeg (Black arrow) and elastin fibers (Orange arrow), B- (original magnification x 100) the same section.

In the above slides, the epithelium is exhibiting the purple staining. Fibroblasts and inflammatory cells are visible in the same color. Collagen bundles appear to be condensed/ hydrated in a blue color. Elastin fibers are detected as tiny dark green fibers. We can also observe red blood cells in the vessels.

11-Immunohistochemical Analysis:

11-1 CD44 (The hyaluronan receptor):

The following scores were used as a criterion in evaluation of tissue sections stained withCD44 antibody, which detects hyaluronan receptors that are predominantly present on macrophages.

- Degree 0= No stained cells detected or the minimum amount
- Degree 1= Small numbers of stained cells
- Degree 2= Moderate numbers of stained cells
- Degree 3= Many stained cells

Figure 4 shows the above scores used to describe the number of stained cells.

Fig 4- A

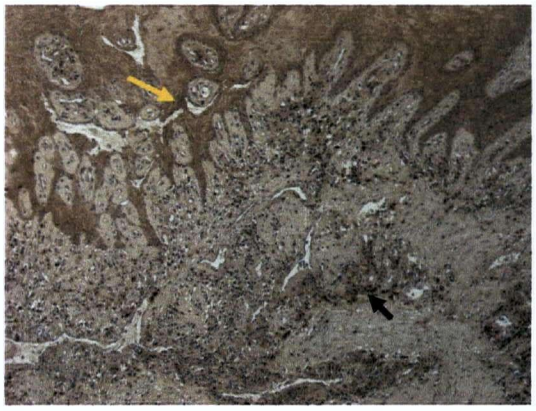

Fig 4- B

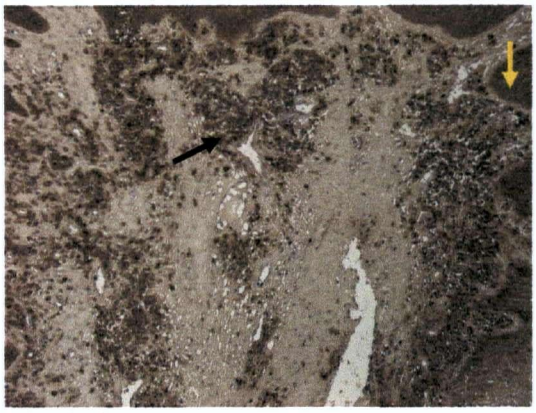

Fig 4- C

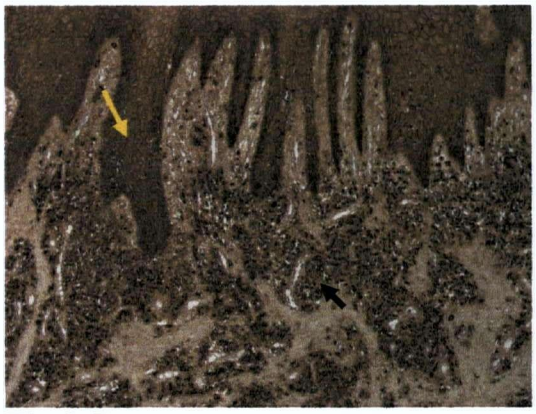

Fig 4- D

Figure 4 - Tissue sections stained with CD44 antibody. A- Absence of stained macrophages, B- Mild accumulation of macrophages, C- Moderate accumulation of macrophages, D- Many CD44+ macrophages. Black arrow points the stained cells. In these slides epithelium shows the similar antigenicity and demonstrates staining in all slides (yellow arrow) original magnification of all slides x 100.

11-2 2B1- Versican Staining

A similar index was made to classify staining for the core protein of versican (using monoclonal antibody 2B1) as follows:

- Degree 0= No stained areas are detected
- Degree 1= Mild accumulation of versican
- Degree 2= Moderate accumulation of versican
- Degree 3= Severe accumulation of versican

Figure 5 shows different degrees of staining:

Fig 5-A

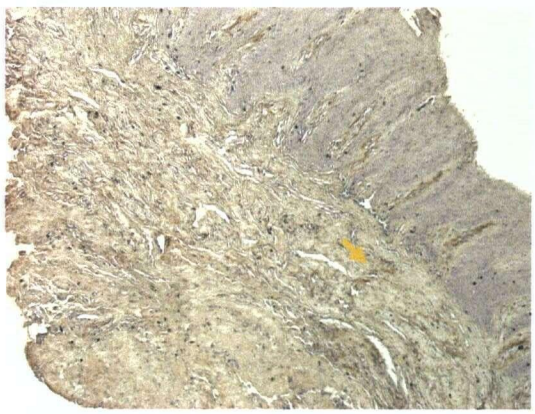

Fig 5- B

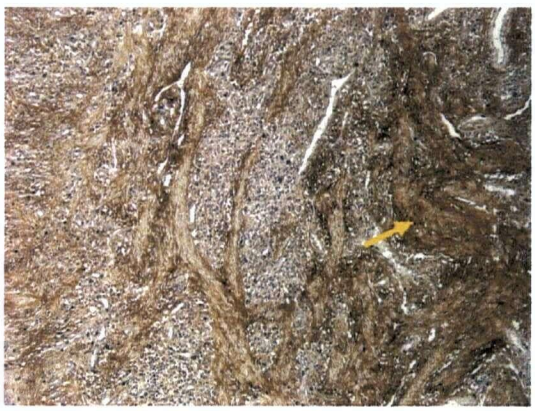

Fig 5-C

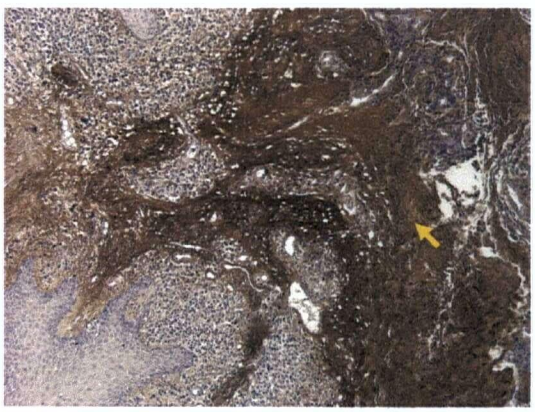

Fig 5- D

Figure 5– 2B1 staining of tissue samples demonstrating different degrees of accumulation of versican in connective tissue. A- Absence of versican, B- Mild accumulation of versican, C- moderate accumulation of versican, D- Severe accumulation of versican. Yellow arrow points Stained areas (original magnification x 100)

11-3 LC2- Versican Staining:

In these slides the C-terminal of versican proteins was subjected to staining. The result of this staining did not lead us to make any scoring, since it was not as prominent as what we observed with 2B1.

11-4 1A4- α-Smooth muscle actin:

To analyze the slides stained with this antibody, in addition to the degree of vascularization, presence or absence of myofibroblasts were determined.

11-5 PCNA- Proliferating cells:

Instead of using any scores to analyze these slides, the number of stained cells, which were mainly located in the basal layer, was quantified using a cell counter. The counting procedure was performed twice for each slide, and in case of any discrepancy the mean was calculated. Results expressed as numbers of positive stained cells/ 200 cells (in a random field).

12-Statistical Analysis:

Mean and SD were calculated for variables such as age, dose and serum level of CSA, PD, OHI and GI. T-student test performed to reveal the degree of similarities between the variables in control and DGO group. To compare the findings in 2 groups, Student's t-test was used to compare the 2 groups; statistically significant differences were considered if p-values of 0.05 or less were calculated.

Chapter 4- Results:

1- Clinical Findings:

In the gingival overgrowth group there were 8 women and 12 men with an age range of 16 to 50 years. The clinical measurements made in this group including probing depth (PD), oral hygiene (OH), gingival index (GI) in addition to the dose and serum level of cyclosporine and number of years after transplant are shown in table 2. The etiology of renal failure and subsequent transplant in most of the cases was unknown. In 3 cases some kind of congenital deficiencies were mentioned (usually in younger patients) and in one case renal failure had happened secondary to hypertension but for the other 14 patients, even in the medical chart there was no certain etiology specified. Most of individuals in gingival overgrowth group were taking some other medications in addition to CSA, such as prednisone 5-10 mg/day, and azathioprine (Imuran) 50-100 mg/day. Azathioprine produces immunosuppression by inhibiting purine synthesis in cells, thereby preventing RNA and DNA synthesis. It is prescribed in renal and bone marrow transplants, and autoimmune diseases such as pemphigus (Gage & Pickett 2001). Two patients were also taking nifedipine for their hypertension (patients # 2 & 5).

Clinical oral examination in this group also revealed changes in gingival color, consistency, texture, contour and form. The changes were localized to facial aspects of maxillary and mandibular anterior segments in most of the cases. In a few cases some changes were observed on the buccal surfaces of posterior teeth, which were not as severe as the anterior ones. Overgrowth generally occurred in areas, where teeth were present. We did not recognize any tissue changes in those edentulous patients wearing complete dentures.

Photographs were taken to show the clinical changes (Figs 6-11) and the manifestations are discussed in below:

Changes in Color: Almost all of the patients showed erythematous changes in gingiva. In some instances a bluish red color was observed.

Changes in consistency: The overgrown tissues were soft, spongy and extremely fragile in most of patients. In addition, the tissue bled easily upon probing. These patients were also complaining about spontaneous bleeding or bleeding upon brushing. In a few cases, fibrotic changes were observed (Figure 10) and the tissue seemed to be firm and resilient.

Changes in texture: Loss of stippling was observed in all cases. The gingival surface was generally shiny and nodular.

Changes in contour and form: In most of the cases both papillary and marginal gingiva were involved. In many instances the papillary and the marginal gingiva were united and developed into a massive tissue fold covering a considerable portion of the crowns.

The first post-operation images are shown in Figures 12 and 13.

Table 2- Demographic and clinical findings in gingival overgrowth patients

Data / Patients	Sex	Age	Dose of CSA mg/ day	CSA serum level ng/ml	Years of kidney transplant	Dose of Prednisone Mg/day	Other drugs	PD	OH	GI
1	M	16	225	135	2 years	5	-	3.9	40%	1.8
2	F	21	200	85	9 years	10	Imuran-Nif	3.9	65%	1.8
3	M	35	275	144	2 years	10	-	5.1	60%	1.6
4	M	36	225	125	2 years	10	-	4.8	50%	2.0
5	M	17	250	150	4 years	5	Imuran-Nif	4.3	66%	2.6
6	F	25	200	125	4 years	5	-	3.9	75%	1.9
7	M	30	250	135	4 years	5	-	5.2	29%	2.6
8	F	22	250	135	3 years	10	-	3.6	55%	1.7
9	F	25	200	170	9 years	5	-	4.6	66%	2.0
10	M	32	150	106	6 years	10	Imuran	3.9	74%	1.9
11	M	17	200	125	4 years	5	-	3.9	55%	2.5
12	M	30	200	128	2 years	10	-	5.3	50%	2.5
13	M	16	150	183	2 years	5	Imuran	4.9	45%	2.7
14	M	21	300	162	8 years	10	Imuran	4.8	37%	2.5
15	F	24	250	136	1 year	5	Imuran	3.9	50%	2.3
16	M	34	275	155	8 years	5	Atenolol	5.9	62%	2.5
17	M	50	225	148	4 years	10	-	4.8	40%	2.2
18	F	42	200	NM	3 years	5	-	5.3	50%	2.2
Range	Σ F= 6	16 -50	150 - 300	85- 183	1-9	5-10	6 Imuran	3.6- 5.9	29-75	1.6-2.7
Mean ± SD	ΣM=12	27.4± 9.5	223.6± 40.46	138± 23.27	4.3± 2.6	6.6± 2.4	1Atenolol 2 Nifedipine	4.5± 0.6	54± 12.8	2.2± 0.35

NM= Not mentioned PD= Probing depth GI= Gingival index OH= Oral hygiene effectiveness

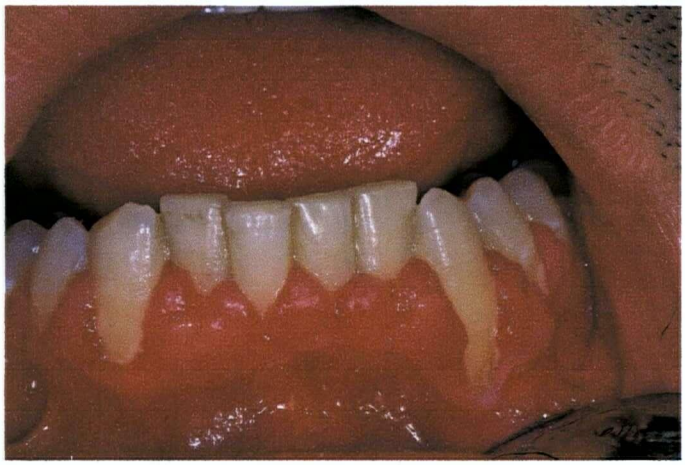

Figure 6- Patient # 12, GOI II

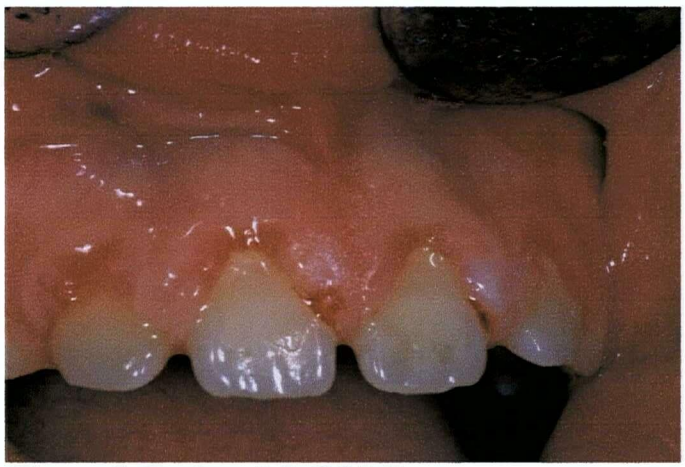

Figure 7- Patient # 5, GOI II

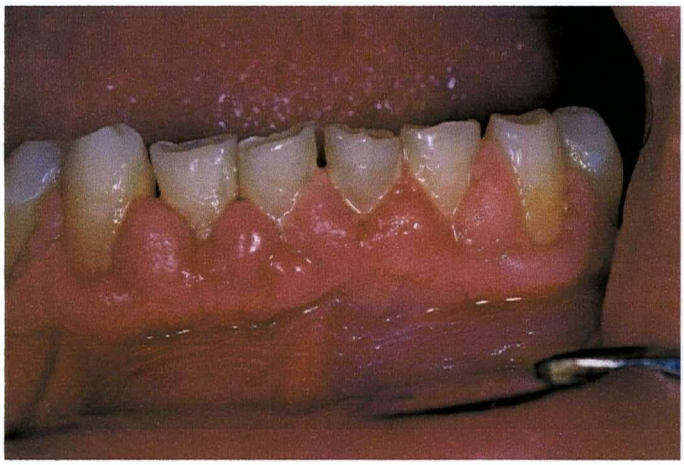

Figure 8- Patient # 17 GOI II

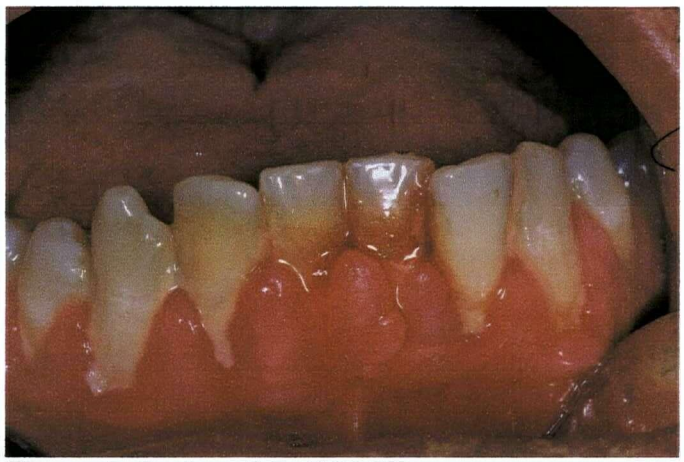

Figure 9- Patient # 7, GOI III

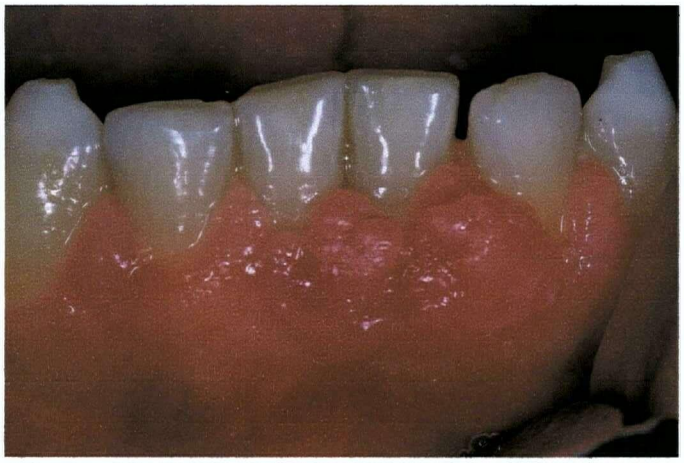

Figure 10- Patient # 16, GOI III

In all above pictures we can observe dramatic changes in gingiva. Papillae are lobulated and cover a part of the crown. Gingival margins do not exhibit the knife-edge shape. The tendency toward bleeding (on probing or spontaneously) can be visualized. All of the pictures except fig 10 demonstrate erythematous changes. In patient #16 (fig 10) the tissue is more fibrotic compared to the other cases and patient reported of having gingival surgery once and recurrence of the overgrowth in less than one year but in a less degree of severity. We can see spacing between the teeth in some of the cases. It was asked of the patients whether they were aware about the onset of the spacing, which in some cases was attributed to gingival overgrowth.

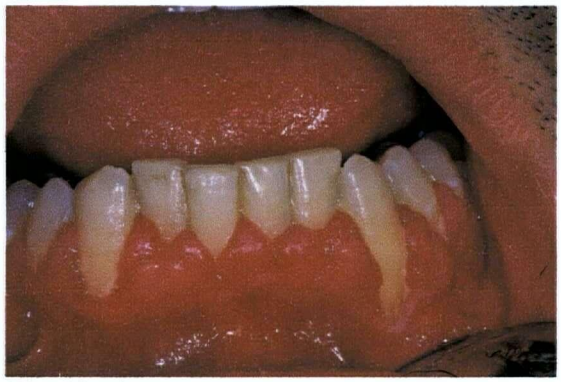

Fig 12- A

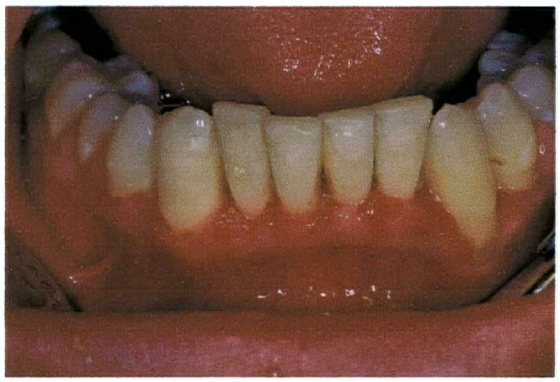

Fig 12- B

Figure 12- Patient #12- A- Before operation, B- One week post-op

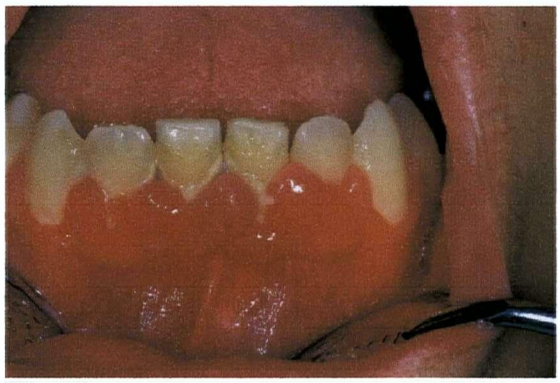

Fig 13- A

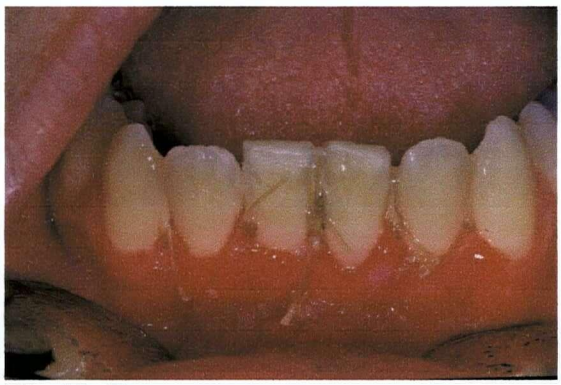

Fig 13- B

Figure 13- Patient # 13- A- Before operation, B- one week post-op

In one week post-op we still can observe some degrees of inflammation, which is not unusual in periodontal surgeries. The excess tissue is removed and we can see the normal length of the clinical crown. Patients were instructed to perform oral hygiene at this stage again. Regarding mucogingival problems, patient # 12 was referred to SHB dental school, Periodontics Department to receive other necessary treatments. In none of the cases did we have any problem with healing process. We did not observe any case with infection or delay in healing.

Demographic findings in control group including sex and age in addition to clinical findings are shown in table 3. As previously stated, control group was systemically healthy and the individuals were not taking any kind of medications.

There was only 1 smoker in each group and because of this low number it is not shown in the tables.

Table 3- Demographic and clinical findings in control group

Data / Patients	Sex	Age	PD mm	OH %	GI
1	F	22	2.3	70%	1.2
2	F	38	2.1	50%	1.4
3	M	36	3.2	65%	1.3
4	F	24	3.1	50%	1.9
5	M	25	3.7	45%	2.1
6	M	36	2.9	75%	1.8
7	M	22	2.6	60%	2.0
8	M	52	2.2	70%	1.5
9	F	39	3.4	85%	1.4
10	M	45	3.1	75%	1.9
11	M	32	3.1	45%	2.2
12	M	30	3.5	50%	1.7
Range	Σ F= 4	22-52	2.1-3.7	50-85%	1.2-2.2
Mean±SD	Σ M=8	33.4±9.4	2.9±0.52	61.6±13.5	1.7±0.33

As we can observe in tables 2 and 3, the proportion of female/male in both groups is the same. The groups were age-matched and t-student test did not show any statistically significant difference between them. With OH effectiveness also we did not detect any significant difference between two groups. Regarding probing depth and gingival index, the overgrowth group exhibited a statistically significant higher scores compared to the control group, which is expected. Table 4 shows the statistical analysis regarding the demographic and clinical findings between 2 groups.

Table 4- Statistical analysis of the demographic and clinical findings between DGO and control groups.

Variables / Groups	Sex	Age Mean± SD	PD mm Mean± SD	OH % Mean± SD	GI Mean± SD
Control group	F= 4 M= 8	33.4± 9.4	2.9± 0.52	61.6 ± 13.5	1.7± 0.33
DGO group	F= 6 M= 12	27.4± 9.5	4.5± 0.6	54 ± 12.8	2.2± 0.35
T-student test	F/M= 1/2	T= 1.7	T= 8	T= 1.6	T= 3.5
P-value		$P \leq 0.1$	$P \leq 0.001$	$P \leq 0.1$	$P \leq 0.01$

2-Findings in Epithelium

2-1: H&E staining: We compared gingival overgrowth to control samples. In the gingival overgrowth group, mostly a thick stratified squamous epithelium was covering the underlying connective tissue. The retepegs were elongated, either thin or thick and were extended deeply into the connective tissue. Based on the comparison with the control group and normal gingiva histologic pictures, we determined the retepegs dimensional changes. The number of retepegs was also increased in the GO group and we observed the proliferation of the basal layer. In some areas, confluent retepegs were visualized. We can observe confluent retepegs when following elongation, they join each other. Signs of hyperkeratosis, parakeratosis and acanthosis were observed in most of the cases in this group (Fig 14 and table 5). These terms are defined as followings:

- Hyperkeratosis: Excessively thickened layer of the stratum corneum.
- Parakeratosis: Presence of residual nuclei in keratin layer
- Acanthosis: Excessive thickening of the spinous layer of squamous epithelium, resulting in broadening and elongation of the retepegs (Sapp et al 2004).

Table 5- Summary of histological findings in epithelium of GO and control groups.

Groups / Histological Features			Control n=12	Gingival Overgrowth n=18
Epithelial Changes	Retepegs	longer	0/ 12	12/18
		Thicker	4/ 12	9/ 18
	Acanthosis		2/12	10/18
	Hyperkeratosis		1/12	5/18
	Parakeratosis		1/12	12/18

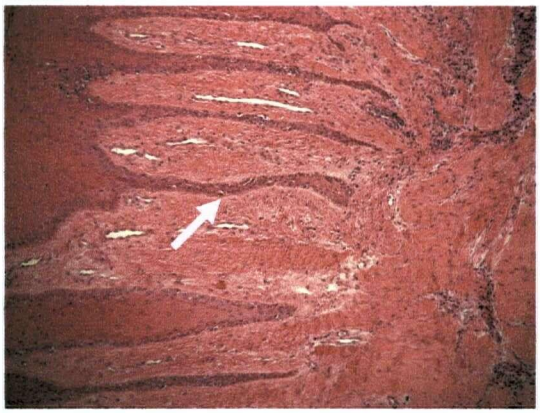

Fig 14- A

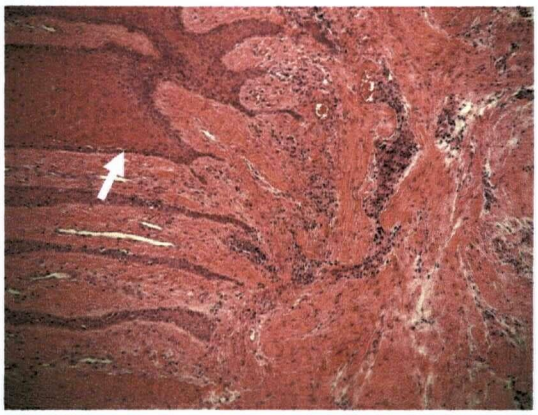

Fig 14- B

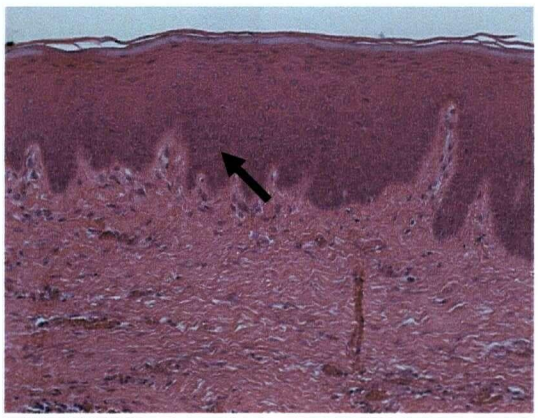

Fig 14- C

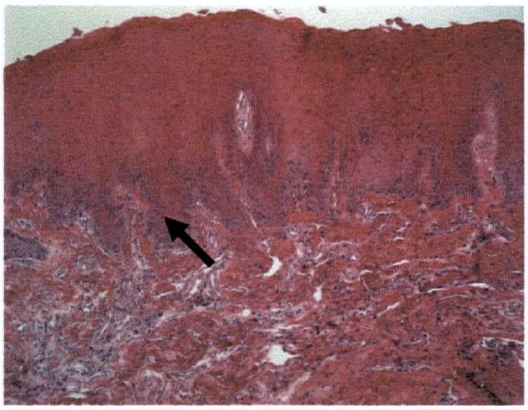

Fig 14- D

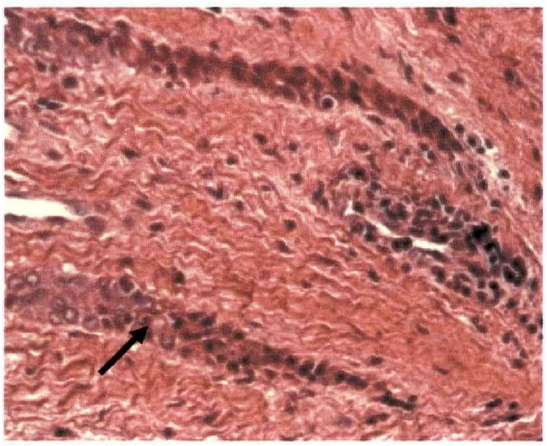

Fig 14- E

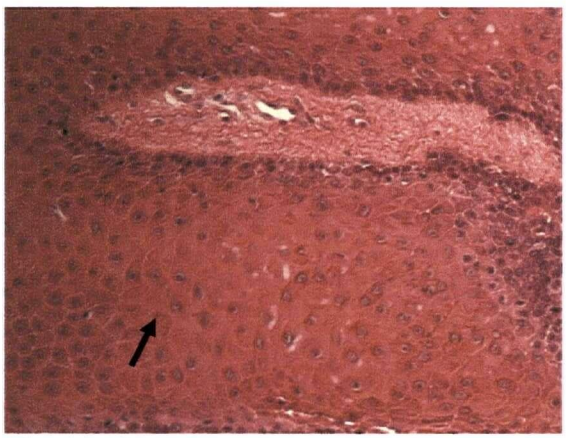

Fig 14- F

Figure 14 – Hematoxylin and Eosin staining- A, B (original magnification x 10)-Long and thin retepegs penetrated deeply into connective tissue in gingival overgrown tissue (white arrow points elongated retepegs) C, D (original magnification x 100)-Normal gingival specimens demonstrating normal retepegs (black arrow shows the retepegs), E (magnification x 300)- Elongated retepegs in gingival overgrowth tissue(black arrow), F (magnification x 200)- Note acanthosis (black arrow)

2-2 PCNA staining

PCNA antibody was used to stain the proliferating cells. Eighteen overgrowth and 11 normal samples were stained with concentrations of 1/100, 1/200, 1/500 and 1/1000. Three slides remained as controls. In all the slides one area was randomly selected and the number of positive stained cells out of 200 cells was counted. The differences between 2 groups were then compared. Staining as expected was more prevalent in the basal layer cells in both groups. Comparison between 2 groups revealed that gingival overgrowth samples demonstrate more stained cells. This finding is in agreement with the changes we observed in H&E stained sections, which the latter also demonstrated proliferative changes in retepegs of GO group. Although most of the stained cells are observed in the basal layer, we could detect some stained cells in the spinous layer, as well. The presence of staining in this layer can be a result of quick turnover, which is probably because of proliferation.

Fig 15 and table 6 show the differences in the number of stained cells in both groups.

Table 6- Statistical analysis of PCNA stained cells in the control and GO group

Stained cells/200 cells / Groups	Mean	SD
Control	61	37.5
GO	126	31.5
Student- t	T= 5.3	
P-value	$P \leq 0.01$	

Fig 15-A

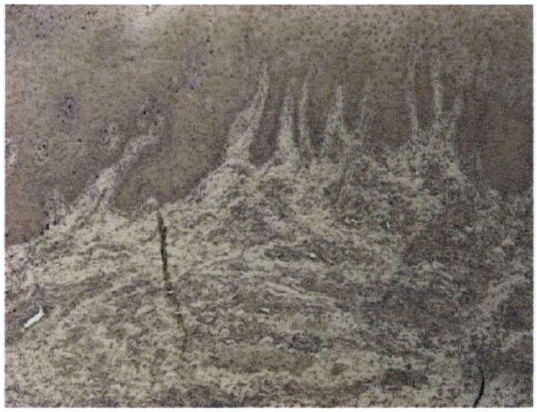

Fig 15-B

Fig 15- C

Fig 15- D

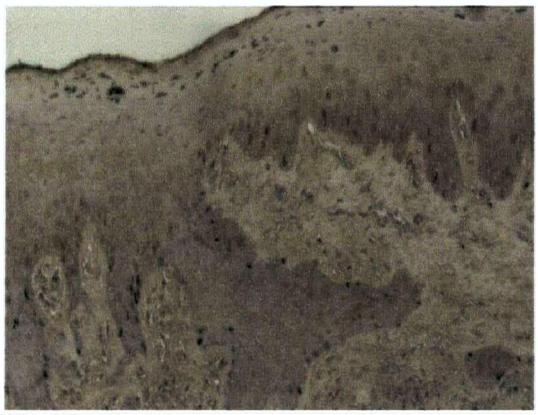

Fig 15- E

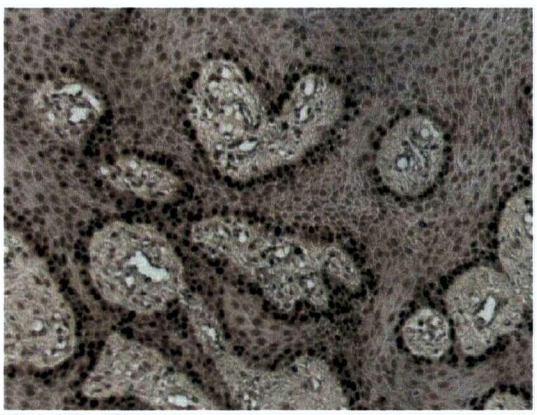

Fig 15- F

Fig 15- Staining with PCNA in the basal layer of epithelium in both groups. A, B (original magnification x 100) - normal tissue samples demonstrate a lesser number of stained cells. C, D (original magnification x 100) Increased number of stained cells in overgrowth tissue samples can be observed. Orange arrow show the stained cells in basal layer and yellow arrow points the ones in spinous layer. E (magnification x 150) less number of stained cells can be observed in one of the control samples compared to GO samples. F (magnification x 200) more stained cells in GO samples compared to Fig E

3- Findings in Connective Tissue

3-1- H&E Staining: A dense collagenous fibrous connective tissue was observed in most of overgrowth gingival specimens. We observed increased collagen bundles compared to controls. The tissue was expanded in gingival overgrowth group compared to healthy group. Increased number of fibroblasts and inflammatory cells could also be observed in this group. Inflammatory cells were mainly representative of chronic inflammatory infiltration. The degree of vascularization also seemed to be higher compare to the control group (Fig 16). It was also noted that some of vessels exhibited thickened walls, and apparent vasculitis.

3-2 – Gomori Trichrome/ Aldehyde fuchin Staining:

In these slides, the most prominent feature was the bundles of collagen, which demonstrated a deep bluish green color and a wavy course. In some of the areas the collagen fibers appeared dense and in the other areas some empty spaces were observed in between. The other observations included keratinocytes, which appeared purple, vessels with red blood cells in the lumen and elastin fibers looking like tiny purple fibers surrounded by collagen bundles. We also observed perivascular infiltration in some of the features (Fig 17). Since the areas representing the proteoglycans also demonstrate the purple color, it was difficult to differentiate them from other features with the same staining reaction, so, the alcian blue/ picrosirius red staining was performed to get a more precise observation regarding proteoglycans and glycosaminoglycans. Collagen and elastin fibers appeared similar in content and appearance between the diseased and the control groups.

3-3- Alcian blue/ Picrosirius red Staining:

In the slides stained with AB/P red, the red areas are representative of collagen bundles within the connective tissue, which can be either dense or hydrated (in the case latter we can observe white spaces in between). Epithelium in these slides demonstrates the light pink staining (Fig. 18). In some of the cases, between the collagen bundles we can observe some areas stained in light blue which are representative of proteoglycan and glycosaminoglycans. These areas are usually found in deeper parts of connective tissue. As it was mentioned in materials and methods we used a grading scheme, based on that, GAG accumulation was classified as minimum, moderate and severe. Table 7 demonstrates the histological findings in connective tissue in both groups and table 8 shows the statistical analysis between the two groups regarding inflammatory infiltration and GAG accumulation.

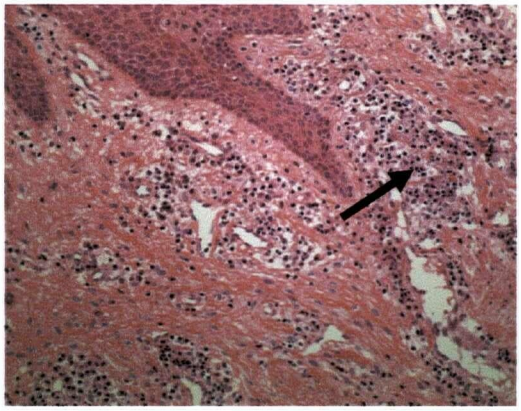

Fig 16- A

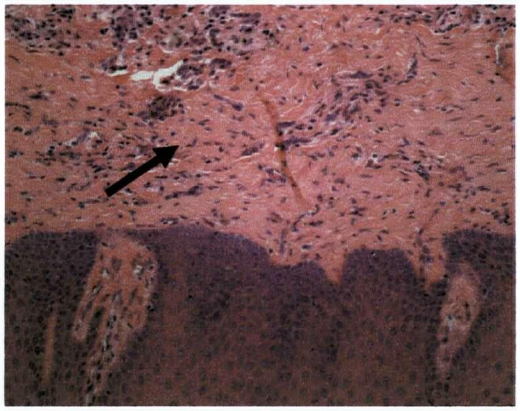

Fig 16- B

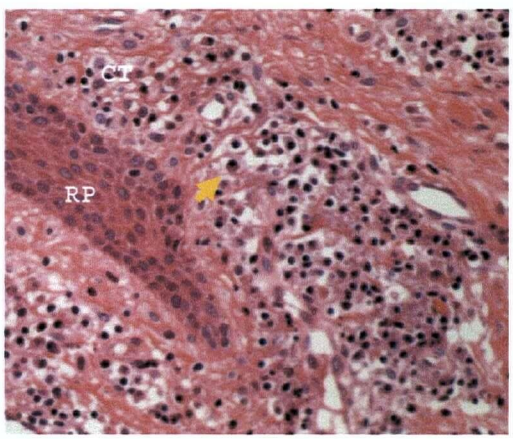

Fig 16-C

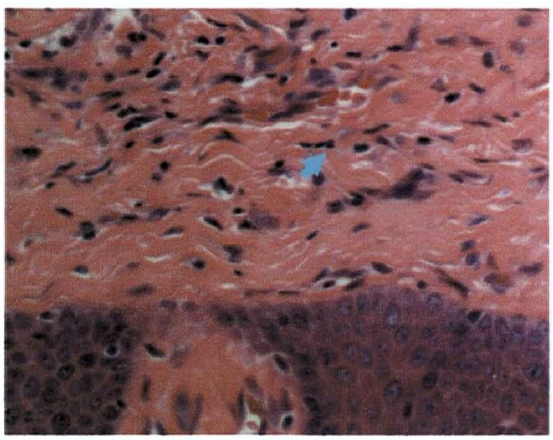

Fig 16-D

Figure 16- Hematoxylin and Eosin staining- A (original magnification x 100)- Severe infiltration of inflammatory cells in gingival overgrowth sample, B (original magnification x 100)- Moderate infiltration of inflammatory cells in control gingival sample, C (magnification x 150)- Inflammatory infiltration in gingival overgrowth sample, mainly composed of plasma cells (arrow) and lymphocytes, D (magnification x 200) Control group sample, most of the cells are fibroblasts (arrow)

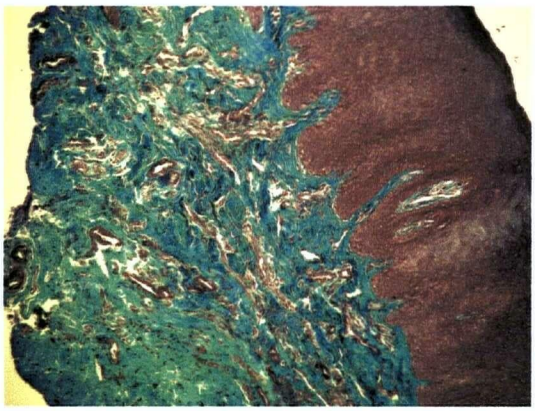

Fig 17-A

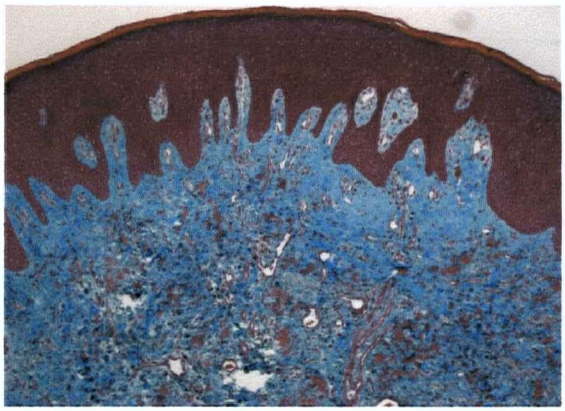

Fig 17-B

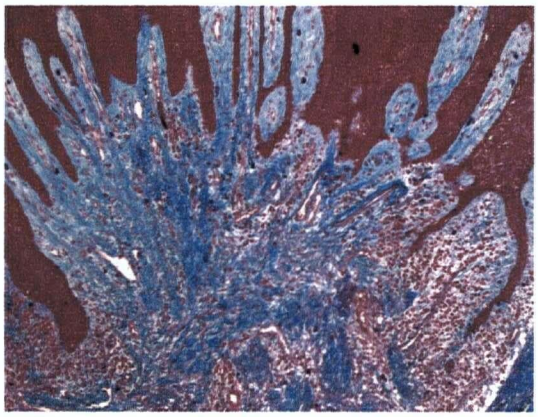

Fig 17- C

Fig 17-D

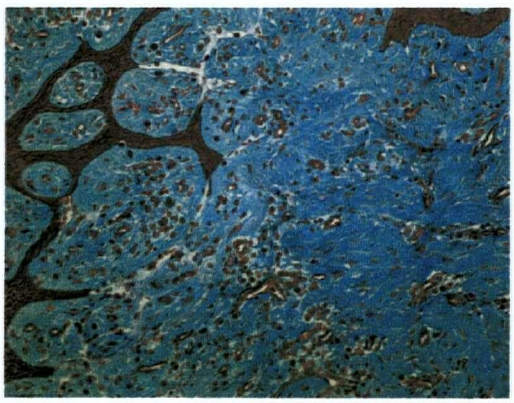

Fig 17-E

Fig 17-F

Fig 17- Gomori Trichrome/ Aldehyde fuchin Staining: A, B- Dense bundles of collagen in control group. C, D, E- Dense collagen bundles in gingival overgrowth group. F - Less density of collagen bundles, blood vessels with RBC and keratinocytes are observed in DGO group. Perivascular infiltration can be observed (arrow). (original magnification of all slides x 100)

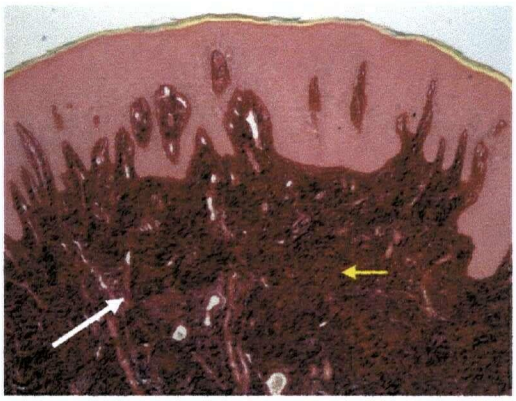

Fig 18-A

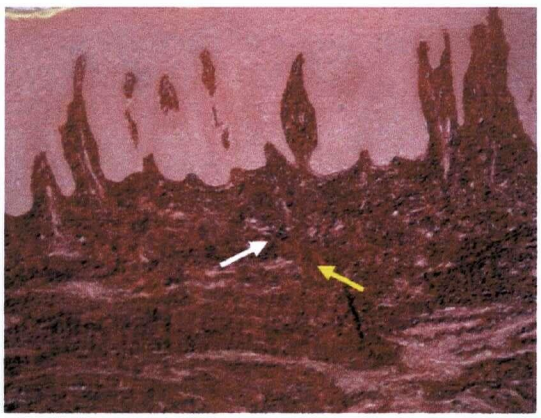

Fig 18-B

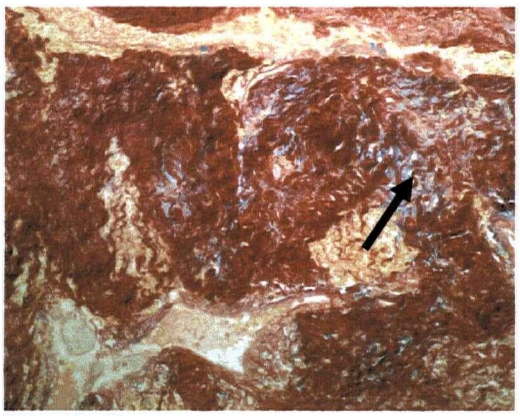

Fig 18-C

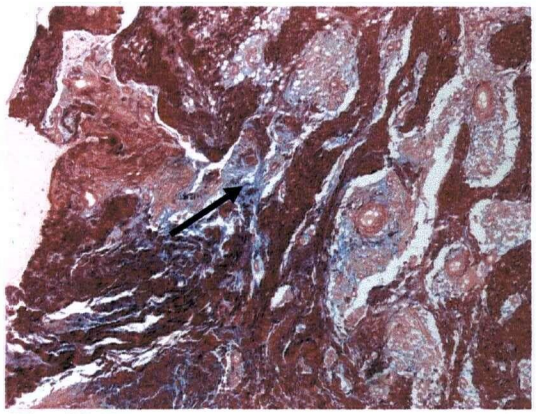

Fig 18-D

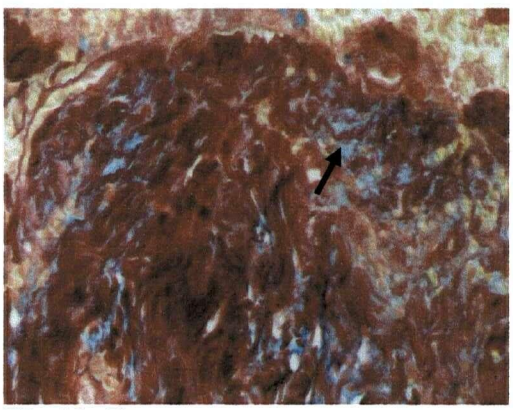

Fig 18- E

Figure 18- Alcian blue/ Picrosirius red staining in control and gingival overgrowth groups. A, B (original magnification x 100) - minimum accumulation of GAG in control group. Yellow arrow points the collagen bundles, C (magnification x 150)- moderate accumulation of GAGs in gingival overgrowth group, D (magnification x 150)- severe accumulation of GAGs in gingival overgrowth group. Black arrow points GAGs, E (magnification x 200) - GAG accumulation in gingival overgrowth group.

Table 7- Summary of histochemical findings in the connective tissue of gingival overgrowth and control groups.

Groups / Histological Features			Control n=12	Gingival Overgrowth n=18
Degree of Inflammation Scores 0-3			Mean = 1.4 SD = 0.66 Range = 1-3	Mean = 2.6 SD = 0.48 Range = 2-3
Connective Tissue Changes	**GAG content**		Mean = 0.8 SD = 0.49 Range= 1-2	Mean= 1.5 SD= 0.58 Range=1-2
	Collagen	**Hydrated**	4/12	15/18
		Dense	9/12	4/18

As we can observe in table 7, there are more cases with hydrated collagen in the overgrowth group, which can be attributed to GAG accumulation. In table 8 we can see that both GAG content and inflammatory infiltration in the GO group demonstrate statistically significant higher amounts compared to the control group.

Table 8- Statistical analysis of histochemical findings between GO and control groups.

Variables / Groups	GAG content Mean± SD	Inflammatory infiltration Mean ± SD
Control group	**0.8 ± 0.49**	**1.4 ± 0.66**
GO group	**1.5 ± 0.58**	**2.6 ± 0.48**
T- test	**t = 23**	**t = 30**
P- value	**P ≤ 0.01**	**P ≤ 0.01**

3-4 α-smooth muscle actin - A total of 28 slides (10 healthy, 18 gingival overgrowth) were stained with 1A4 antibody in 2 of experiment. In each round of staining 4 slides (both healthy and gingival overgrowth) were kept as controls, not receiving the primary antibody. 1A4 targets the α-smooth muscle actin, which can be found in vessel walls and myofibroblasts. In all specimens, overgrowth and healthy gingival tissues, vessel walls were stained (brown color). The staining demonstrated more vascularization in overgrowth tissues was more prominent in this group. In addition, some areas representative of myofibroblasts could be observed in overgrowth group. These cells appeared to be in the matrix rather than blood vessel walls and their cytoplasms were stained. We could not detect myofibroblasts in the control group. In Figure 19 we can see the differences between the number and the size of blood vessels in overgrown and normal tissues in addition to presence of myofibroblasts.

3-5 Core of versican antibody:

2B1 antibody was used to stain the core of versican. A total of 30 slides (18 overgrowth samples, 12 normal samples) were stained with this antibody and 6 slides were served as controls. We observed that all of the overgrowth tissue samples demonstrate a higher degree of staining (brown color), which was distributed almost evenly throughout the connective tissue. In normal tissue samples, we observed some degree of staining, which was significantly less compared to the gingival overgrowth group (figure 20). Based on this finding, we concluded that versican is in a part responsible for the dimensional changes, which happens in CSA-induced GO.

Fig 19- A

Fig 19-B

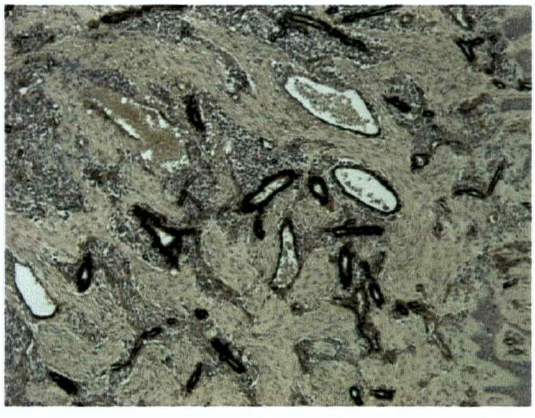

Fig 19-C

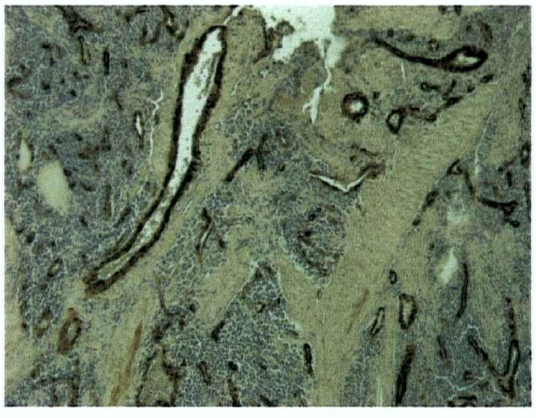

Fig 19-D

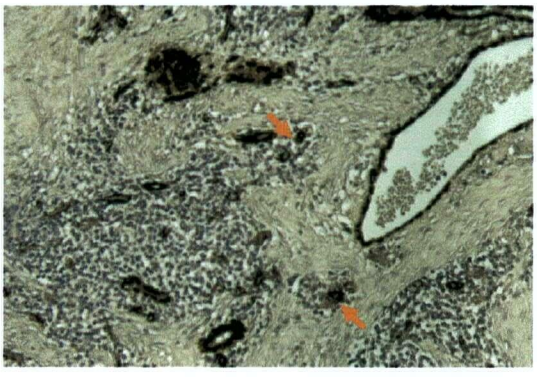

Fig 19- E

Fig 19- Staining with 1A4 to demonstrating α -smooth muscle actin (in blood vessels and myofibroblasts). Fig A, B (original magnification x 100) - Blood vessels demonstrate the staining in normal tissues. Fig C, D (original magnification x 100) - Increased in number and size of blood vessels in gingival overgrowth tissues compared to normal ones. E (magnification x 150) - Presence of myofibroblasts in gingival overgrowth tissue (orange arrow).

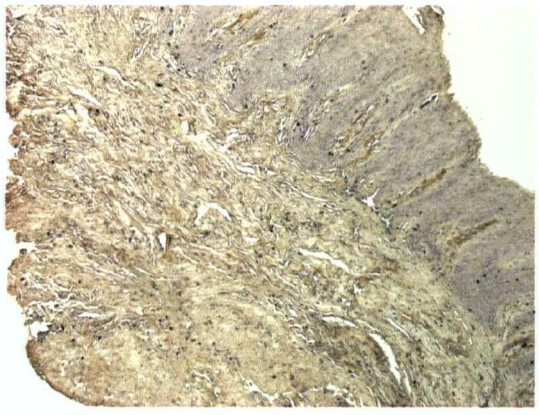

Fig20-A

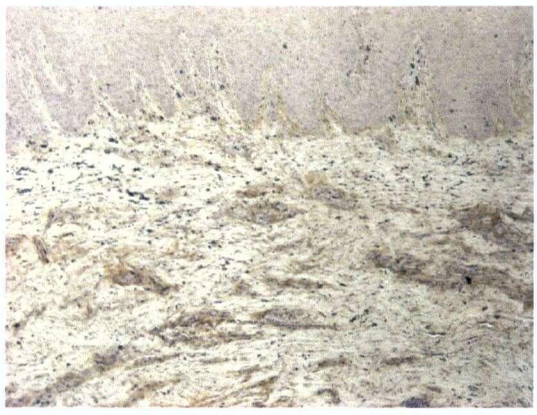

Fig 20-B

Fig 20- C

Fig 20-D

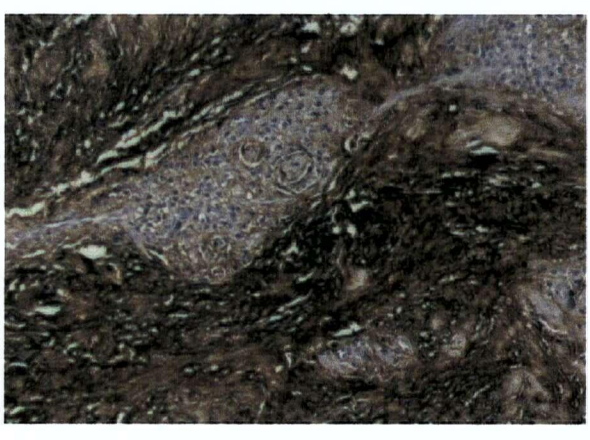

Fig 20- E

Figure 20- Staining with 2B1 antibody to reveal core of versican. Fig A, B (original magnification x 100) - mild accumulation of versican is seen in normal tissues. Fig C, D (original magnification x 100) - Severe accumulation of versican in overgrown tissues. Fig E (magnification x 200) Severe stained areas representative of versican accumulation in gingival overgrowth tissue

3-4 C terminal of Versican:

LC2 antibody was used to stain the C terminal of versican. A total of 36 (20 gingival overgrown and 16 normal samples) slides were treated with this antibody at concentrations of 1/500, 1/1000, 1/5000. 8 slides remained as controls. Although we did not get the same strong staining as what we got with 2B1, we still can notice the differences between two groups, with more accumulation of versican in the overgrowth samples. Using higher concentrations of this antibody did not make a major difference in the amount of staining. Figure 21 shows the differences between 2 groups.

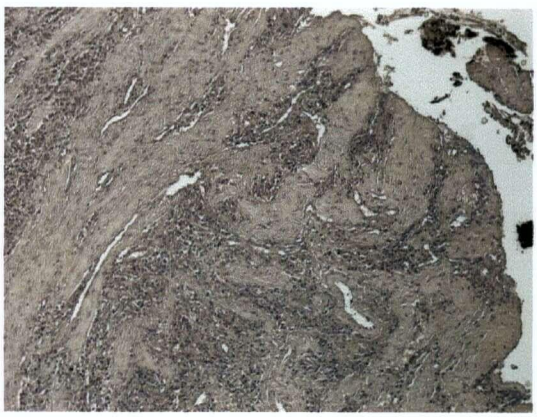

Fig 21-A

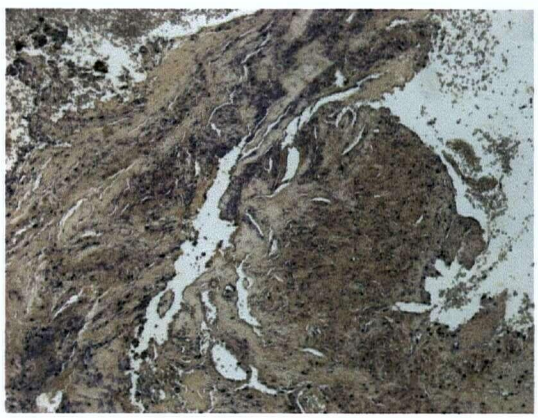

Fig 21-B

Figure 21- A (original magnification x 100) - LC2 staining in normal tissue. B (original magnification x 100) - LC2 staining in overgrown tissue. Although with this antibody a strong staining reaction was not observed, we still can see the difference between the control and diseased tissue.

3-7 CD 44 Staining:

CD44 antibody was used to stain the hyaluronan receptors, which are predominantly on macrophages. Totally, 30 slides (18 diseased and 12 normal samples) were stained with different concentrations of antibody (1/200, 1/500, and 1/1000). 6 slides served as controls and received no primary antibody. All overgrowth tissue samples exhibited more stained cells in connective tissue compared to the controls, which were more prominent in sub epithelial connective tissue. With this antibody, epithelium exhibited the same staining. Between the stained cells, we could differentiate macrophages considering their prominent nuclei. We could also observe some plasma cells. The different degrees of staining in 2 groups are shown in Fig 22.

Table 9 demonstrates the statistical analysis.

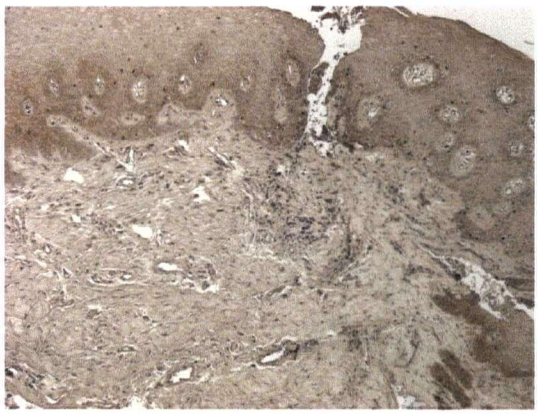

Fig 22-A

Fig 22-B

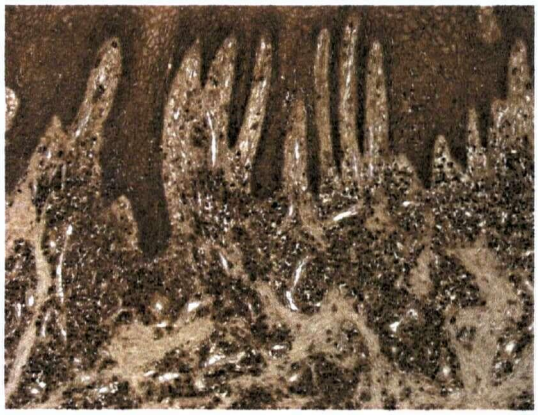

Fig 22-C

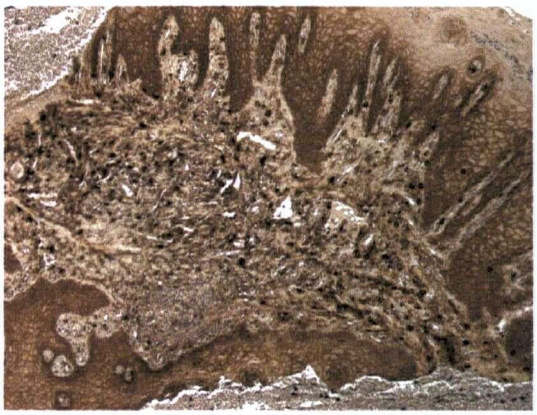

Fig 22-D

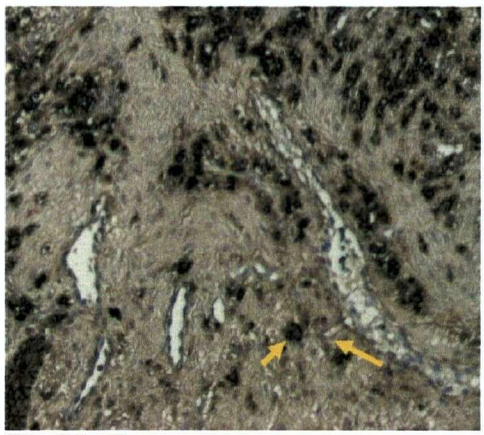

Fig 22- E

Fig 22- Staining with CD44 in both overgrowth and normal gingival samples. A, B (original magnification x 100) - mild accumulation of the stained cells in the connective tissue of normal samples. C, D (original magnification x 100) - moderate and severe accumulation of staining in overgrowth gingival samples. E (magnification x 200) – moderate staining in overgrowth gingival sample. Arrow points the macrophages. Epithelium exhibits the similar antigenicity in all the slides.

Table 9- Statistical analysis of immunohistochemical findings in the GO and control groups.

Groups / Scores	CD44 Mean± SD	2B1 Mean ± SD
Control group	1.08 ± 0.5	1.25 ± 0.45
GO group	1.88 ± 0.67	2.27± 0.66
T- student	T= 3.8	T= 25
P-value	$P \leq 0.01$	$P \leq 0.01$

According to the above table, we have statistically significant higher amounts of staining with CD44 and 2B1 in the DGO group compared to the control group. We can see that the greatest difference happens with 2B1.

Co-Localization:

To compare different stainings in the same slides and see how the changes are related together, figures 23, 24 and 25 show the co-localizations of various stainings in 3 patients with gingival overgrowth. In Figure 23, we can observe that the same areas in Alcian/blue stained slides demonstrate the staining in those slides stained with 2B1, both indicating the presence of GAGs. In Figure 24, the same areas show the staining reaction with LC2, 2B1 and Alcian/blue, which again demonstrate correspondence between the staining for glycosaminoglycans and staining for versican. In figure 25, we see that same areas in the slides stained with Alcian/blue and 2B1 exhibit the accumulation of GAGs, while in the slide stained with LC2 we see less amount of staining in that area. As LC2 is raised against C-terminal of versican, that may means versican in tissues has been proteolytically processed and lacks the C-terminal domain.

Fig 23-A

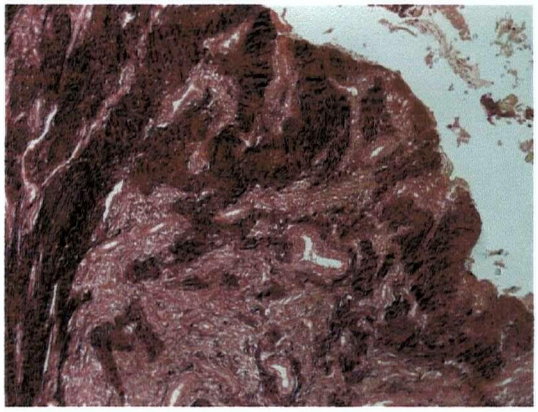

Fig 23-B

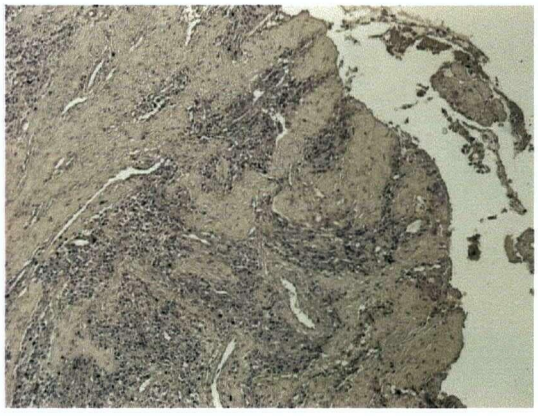

Fig 23-C

Fig 23- D

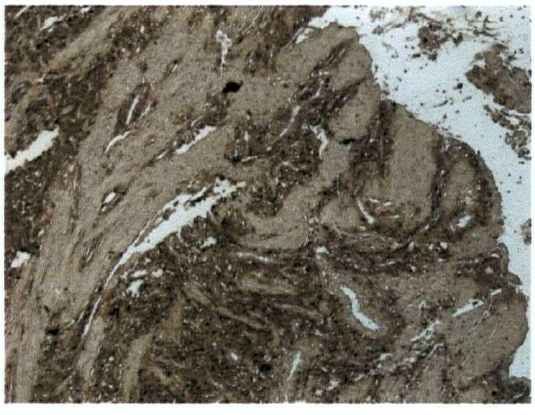

Fig 23- E

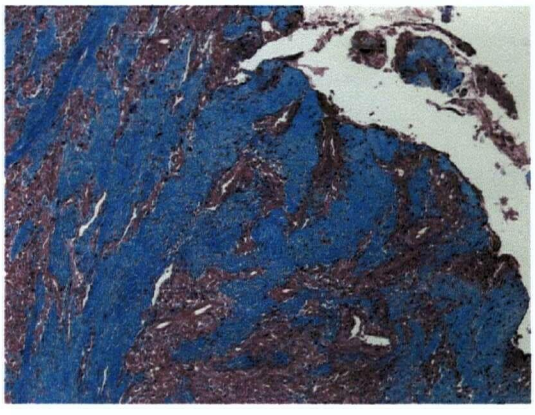

Fig 23- F

Fig 23- Co-Localization of different stainings in patient # 6- A- Staining with 1A4, B- Staining with Alcian/blue, C- Control specimen, D- Staining with 2B1, E- CD44 staining, F- Gomori/ trichomorious staining (original magnification x 100 in all the slides).

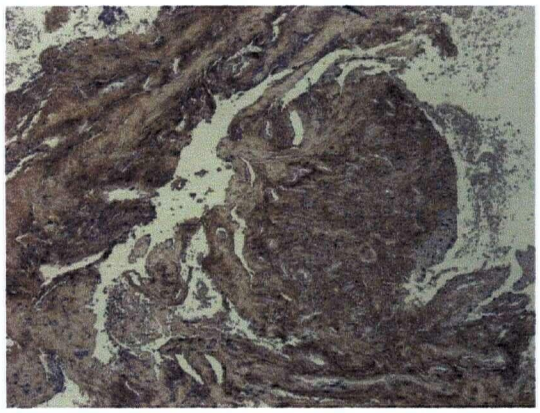

Fig 24-A

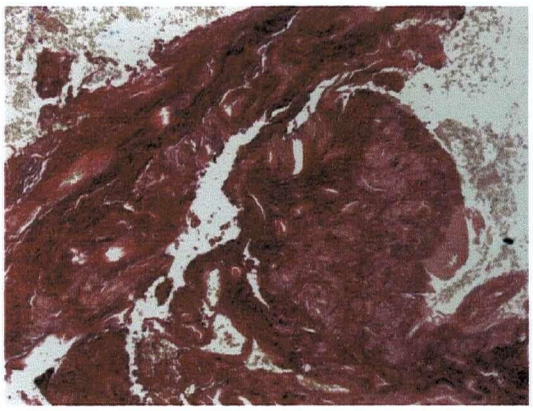

Fig 24-B

Fig 24-C

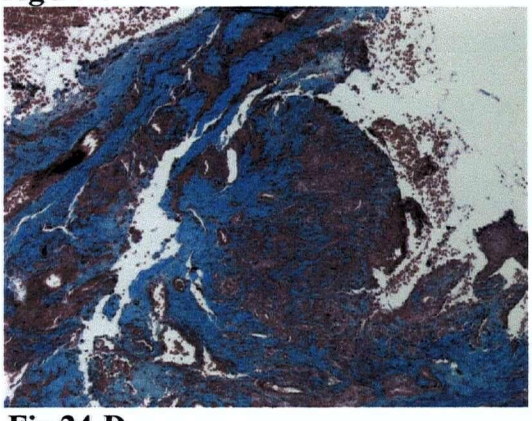

Fig 24-D

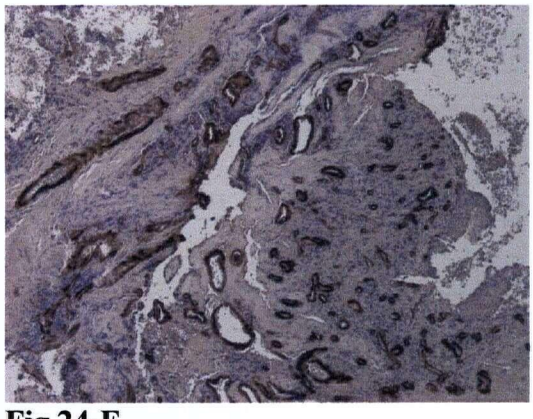

Fig 24-E

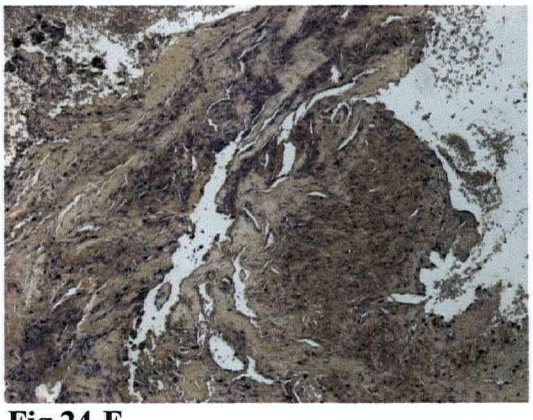

Fig 24-F

Fig 24- Co-Localization of the same sections with different stainings in patient # 10. A- 2B1 staining B- Alcian/Blue staining, C- CD44 staining, D- Gomori staining, E- 1A4 staining, F- LC2 staining. (original magnification x 100 in all slides).

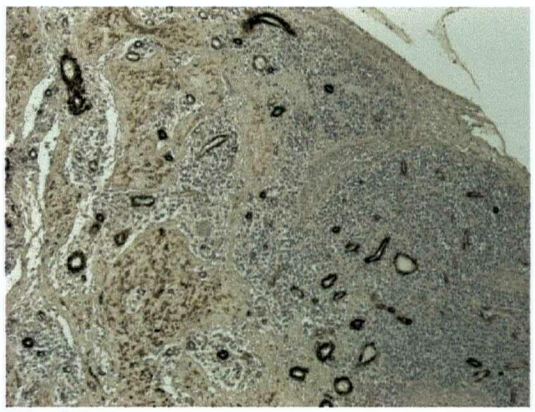

Fig 25-A

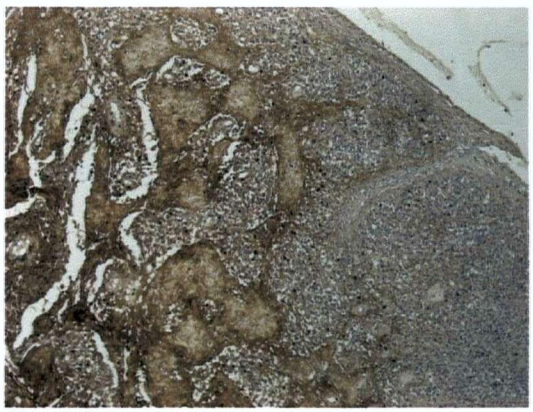

Fig 25-B

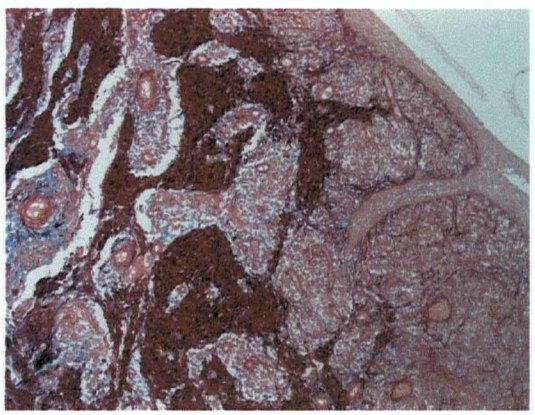

Fig 25-C

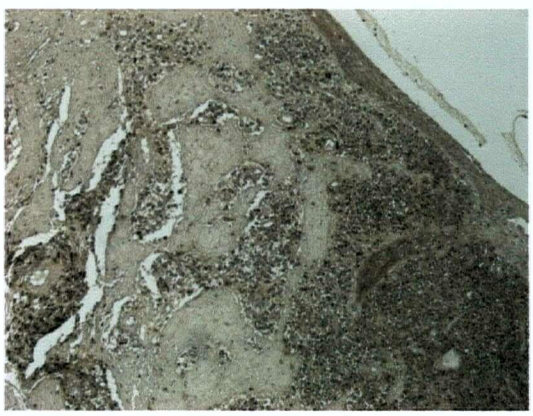

Fig 25-D

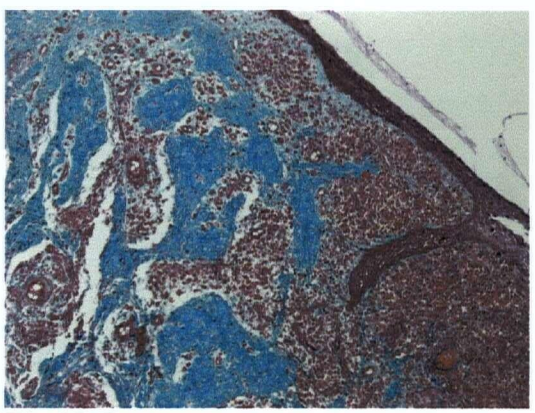

Fig 25-E

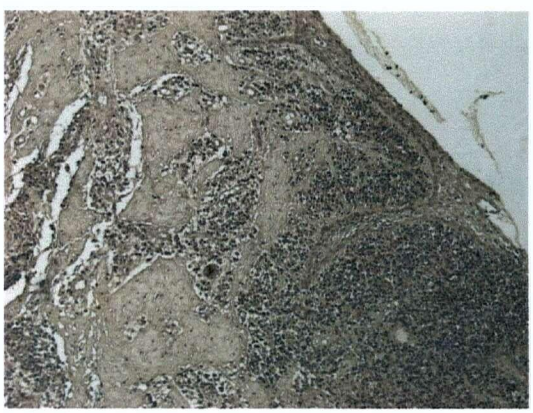

Fig 25-F

Figure 25- Co-localization of the same slides and different stainings. A- 1A4 staining, B- 2B1 staining, C- Alcian/blue staining, D- CD44 staining, E-Gomori staining, F- LC2 staining (original magnification x 100 in all slides)

Chapter 5- Discussion:

The major new findings of this study are as follows:

- Observation of Myofibroblasts in connective tissue of GO group
- Detection of versican accumulation in Connective tissue of GO group
- Increased number of proliferating cells in the basal layer of the epithelium, in GO group

1-Comparison of the findings in the epithelium of overgrowth gingival samples to the previous findings in literature:

H & E staining: H&E stained slides of DGO group revealed significant differences compared to those of control group. We observed a thick stratified squamous epithelium with different degrees of acanthosis and parakeratosis, which penetrates connective tissue with long, thin/thick and irregular retepegs. These observations are in concordance with the findings of Rateitschak *et al* (1983) and Rostock *et al* (1986), who reported an irregular, multilayered parakeratinized epithelium with varying thickness. On the other hand, Rostock *et al* (1986) also mentioned the presence of spongiosis in epithelium, which was not observed in our samples. However, that study was a case report and the biopsies were taken of one African-American male patient. This racial diversity might explain the different features. The SEM study done by Pisanty *et al* (1988) on patients suffering from Behcet syndrome and receiving CSA revealed epithelial acanthosis and the presence of needle like crystallites in epithelium, which were regarded as representing the accumulated and deposited drug. Acanthosis was one of the changes that we observed in most of our cases, however, no crystallite structure was found in the samples, which might be explained by a limitation of light microscopy. O'valle *et al* (1994) observed varying degrees of papilomatosis, acanthosis and epithelial spongiosis in all of their specimens. Meller and colleagues (2002) also report a thickened epithelium and hypertrophy of epithelial cells in rats that received a dose of 40 mg/kg for eight weeks.

Proliferating Cell Nuclear Antibody: With PCNA antibody we observed increased staining in the basal layer of CSA-induced GO group compared to the controls, which indicates active proliferation in that cellular layer (126± 31.5 in GO group versus 61± 31.5 in control group). This finding is in accordance with other studies. Saito *et al* (1999) studying the percentage of Ki-67 to demonstrate the mitotic activity in basal layer of nifedipine-induced overgrown gingiva and compare with the controls, found about 2-fold increases of ki-67 staining percentage in the diseased group. Nurmenniemi *et al* (2001) also compared the mitotic activity of the basal cell layer in drug-induced gingival overgrown samples by using monoclonal antibody for ki-67, and determined the mitotic activities of epithelial cells as percentage of ki-67 labeled cells in relation to total numbers of epithelial cells in the basal layer of oral, oral sulcular and sulcular epithelium. They concluded that the increased epithelial thickness is associated with increased mitotic activity especially in the oral epithelium. On the other hand, one recent study from Bulut *et al* 2004 revealed controversial findings. Using Ki-67, they reported similar proliferative activity in the basal layer cells of the CSA-induced gingival overgrowth and control groups. Based on the periodontal parameters of their study population, the control group has more gingival inflammation as revealed by GI (gingival index). According to Schroeder *et al* (1973) a proliferation of retepegs can be observed in early and established lesion of dentogingival epithelium. Thus, more inflammation in the control group can be responsible for observing the same degree of proliferation in basal layer of two groups.

Based on the findings with PCNA staining which show us more proliferation of the basal layer, we can explain the reason we see dimensional changes in epithelial retepegs of the gingival overgrowth samples.

2-Comparison of the findings in connective tissue of overgrowth gingival samples to previous reports: The changes that we noticed in connective tissue included a high level of vascularization,

irregularly arranged collagen fiber bundles, a significant infiltration of inflammatory cells mainly composed of mononuclear cells and accumulation of amorphous ground substances. Other investigations (Rateitschak *et al* 1983, Rostock *et al* 1986, O'valle *et al* (1994), Seymour & Thomson 1996, Hallmon & Rossmann 1999) confirm these findings. However, there is a controversy regarding the number of fibroblasts. While many authors believe that there is no increase in the number of fibroblasts (Rostock *et al* 1986, Pisanty *et al* 1988), other investigators (Mariani *et al* 1996) report observing a high number of fibroblasts and claim that fibroblasts constituted the predominating cellular elements in the connective tissues. Despite this finding, the authors suggested that the term "hyperplasia" is an inappropriate name for this condition and since the increase in volume of gingiva is brought by abnormal quantity of amorphous substance of the connective tissue, the term should be changed to "dimensional increase of gingival tissue". In the present study, we observed that inflammatory cells constitute the highest number of cell populations and we could not find a drastic difference in the number of fibroblasts between control and DGO groups.

In those slides stained with Alcian blue/picrosirius red we observed that most of DGO group exhibited a significant amount of GAG accumulation in connective tissue. This observation is in agreement with many previous studies. It has been reported by Butler & Butler (1974) that the non-collagenous matrix composed 20% of the dry weight in gingival tissue from phenytoin-induced gingival overgrowth and only 7% in normal tissue. Kantour & Hassal (1983) studying the cultures of gingival fibroblasts from patients taking phenytoin observed that these cells synthesize increased amounts of sulphated glycosaminoglycans (GAG). Pisanty *et al* (1988) also reported the accumulation of non-collagenous extracellular matrix. Suresh *et al* (1992) extracted GAGs from human normal, inflamed and phenytoin-induced gingival overgrowth tissue by proteolysis and alcohol precipitation. They observed that GAGs were decreased in inflammation, while it was increased in the overgrowth cases. Mariani *et*

al (1993) observed a particular abundance of amorphous substance when compared to fibrous material in tissue samples obtained from kidney transplant patients. Their histochemical data showed that overgrowth gingival samples contains increased amounts of both sulphated and non-sulphated GAGs. These authors also noted that the increased number of GAGs in the ground substance leads naturally to a greater amount of bound water, and correspondingly to a higher volume and an increased osmotic pressure in the extracellular matrix, which would produce a water-logged connective tissue, thereby accounting for the gingival edema. Saito *et al* (1996) observed heparan sulfate GAG in the lamina propria of hyperplastic gingival tissue while less immunostaining was observed in the control group. Newell and Erwin (1997) compared GAG and hyaluronan synthesis by fibroblasts derived from normal and CSA-induced overgrown gingival tissue and suggested that a direct promotion of GAG synthesis by gingival fibroblasts in response to CSA may play a role in the pathogenesis of gingival overgrowth. On the other hand, Rocha *et al* (2000) observed that there were no differences on the total and relative amounts of GAG between CSA-induced overgrown gingiva and control group. They analyzed the purified GAGs by agarose gel electrophoresis and observed the presence of chondroitin 4- and 6- sulfate, dermatan sulfate, heparan sulfate and hyaloronic acid to be the same in both normal gingiva and CSA- induced overgrowth. From the same group of authors Martins *et al* (2003), observed no significant difference in the molecular size distribution of hyaluronic acid and sulfated glycosaminoglycans among healthy and drug-induced overgrown gingival tissues. The results we obtained from our study, all indicated that there was a marked difference on GAG accumulation between diseased and control group, as most of the other studies observed the same finding. The reason for the controversy could be due to the different techniques which were applied. The set of slides were stained with Gomori Trichrom/Aldehyde fuchin demonstrated collagen bundles, which were dense or hydrated.

Versican: To study more specifically about GAG accumulation we aimed to determine the amount of versican in connective tissue of the gingival overgrowth group and to compare it to the control group. Using 2B1 and LC2 antibodies we were able to observe versican deposition in tissue samples, with 2B1 giving a better staining response. In most of gingival overgrowth samples there was a higher accumulation of versican compared to those of controls. To our knowledge, this is the first report of versican presence in cyclosporine-induced gingival overgrowth. In the study of Gnoatto *et al* (2003), mRNA expression of the proteoglycans perlecan, decorin, biglycan and versican was analyzed by reverse transverse polymerase chain reaction in CSA-induced gingival overgrown samples. The results revealed an increase in perlecan expression compared to control group. However, other proteoglycans including versican did not show any significant difference with controls.

α-Smooth Muscle Actin: Antibody 4A1 was used to identify the α-smooth muscle actin, which is present in both vessel walls and also myofibroblasts. Because of high vascularization in DGO group, there was a marked staining in vessel walls in that group. As mentioned previously, many studies mention this increase in vascularization and its effects on the general picture of the pathological changes. According to Mariani *et al* (1996), the importance of endothelial cells is that they have a high number of micropynocitotic vesicles in overgrown gingiva, which appear to derive from saccular invaginations of the plasma membrane and have openings on the side of the capillary lumen and the opposite side. The vesicles allow the endothelial cells to actively intervene in the exchange of materials through the capillary walls.

Some of the other features of this staining were that the stained areas indicated the presence of myofibroblasts (cells, which could not be detected in control group).

Yamasaki *et al* (1987) were the first to report the presence of myofibroblasts in tissue samples taken from CSA-induced gingival overgrowth. They reported the presence of cells containing microfilament

bands with semiperiodic dense nodes and nuclear indentations. Dill & Iacopino (1997) performed a transmission electron microscopy study of phenytoin-induced gingival overgrowth and demonstrated the presence of cells with ultrastructure characteristics of myofibroblasts which include; microfilaments, electron-dense bodies associated with filament bundles, vesicular structure in close association with surface membrane, presence of desmosomes and hemidesmosomes and a well developed rough endoplasmic reticulum. The latest study discussing the presence of these cells is the one done by Bullon *et al* (2003), in which they observed myofibroblasts in tissue samples taken from patients on calcium channel blockers. They described myofibroblasts as cells with a very fusiform shape, elongated nuclei and well presented cytoplasmic organelles particularly endoplasmic reticulum. The observation of myofibroblasts is significant regarding the association of these cells with the process of tissue turnover/repair and fibrosis. In a series of studies performed by Roberts and colleagues (1996, 1997, 2001) presence of these cells was noticed in pulmonary fibrosis. According to these studies, myofibroblasts express α-smooth muscle actin and resemble smooth cell muscles ultrastructually. They both synthesize the structural elements and contract the granulation tissue, which the later being an important step in closing the wound.

-CD44: CD44 antibody also demonstrated significant numbers of stained cells in overgrown tissues. These cells were mainly macrophages. The reason that we used this antibody is because of the important potential role that macrophages have in the pathogenesis of gingival overgrowth. According to studies of Iacopino *et al* 1997 and Nurmenniemi *et al* 2002, macrophages are a determinant in pathogenesis of drug-induced gingival overgrowth. Schematic picture in figure 26 shows the potential role of macrophages in drug-induced gingival overgrowth. We observed accumulation of proteoglycans in connective tissue of gingival overgrowth tissue, which in turn can be a result of

activation of myofibroblasts. According to the literature, CSA can have this effect on fibroblast through proliferation of more macrophages and production of more growth factors (PDGF-B, TGF-β). CD44 according to Kajita *et al* (2001) is a multistructural and multifunctional cell adhesion molecule that is involved in cell-cell and cell-matrix interactions. It has been shown to play roles in many important physiological and pathological processes such as lymphocyte homing, T cell activation, wound healing, angiogenesis and metastatic spread of cancer cells.

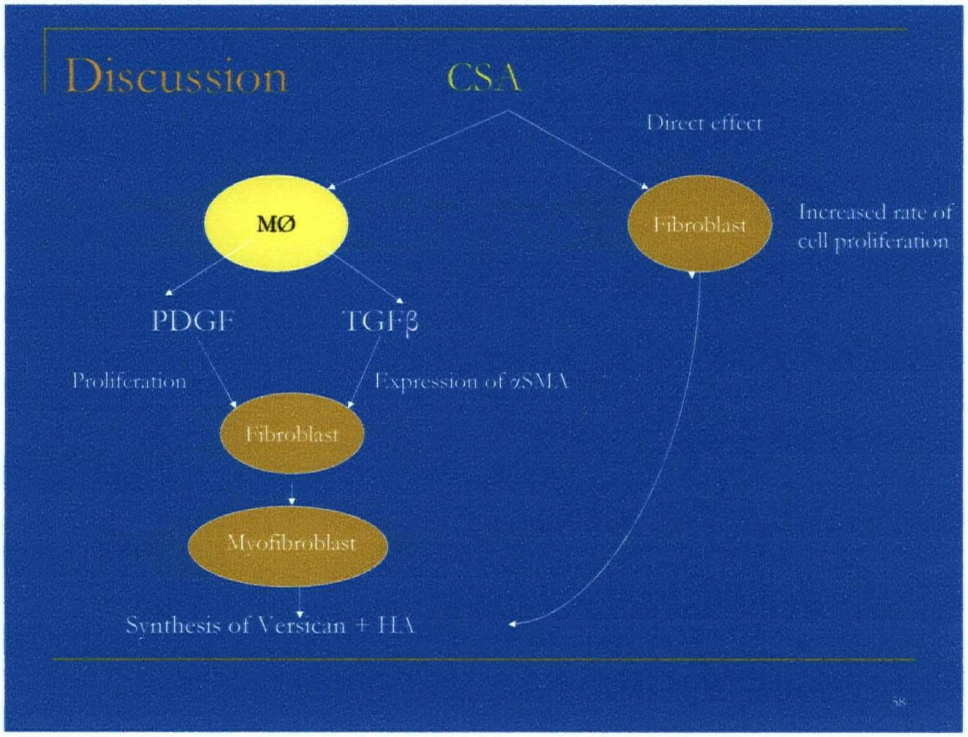

Fig 26- Possible role of macrophages in pathogenesis of DGO

It is clear that gingival overgrowth as a side effect of cyclosporine therapy is driven by both the direct effects of cyclosporine on macrophages and/ or fibroblasts and an effect of altered gingival microflora. As we know that oral hygiene plays a very important part in determining the severity of gingival

overgrowth, it seems probable that there is a positive feedback loop that drives gingival overgrowth in these patients.

I propose that CSA has effects on macrophages and fibroblasts that may cause increased matrix synthesis. At the same time, immunosuppressive effects of CSA may either alter gingival microflora or alter the tissue reaction to the biofilm present in these patients. It is possible that CSA causes both increased matrix synthesis and increased biofilm that drives matrix synthesis leading to a positive feedback loop.

However, despite numerous investigations, which have been done in the field of drug-induced gingival overgrowth, there still are many controversies and many questions to answer regarding issues such as susceptibility, histopathology, pathogenesis, treatment and prevention. Considering the increased number of organ transplants, which are taking place, there is a need to overcome the side effects of these drugs. As a consequence, we still need more studies to increase our knowledge and understand the mechanisms that contribute to tissue overgrowth as a side effect of cyclosporine therapy.

Limitations of the present study:

1- The differences between the overgrown and normal gingival samples were so obvious and clear under the light microscopy, that there was no way to perform this study in a blind way. Where possible, cell counts provided an objective approach to data collection. The scoring systems were confirmed by independent analysis by 2 observers. However, as both observers could clearly see that each sample belonged to one of 2 obvious groups, the possibility of observer bias cannot be discounted extremely.

2- It was not possible to make quantitative measurements. We had to make our own grading schemes for some features, compare the two groups. It is obvious that this qualitative scoring is not a very precise and exact method to describe all the changes.

3- We compared the gingival overgrowth samples with clinically healthy gingival samples. The GO patients exhibited some changes that might be secondry to periodontitis, and that might confound analysis. It would have improved the study if we could have include groups of patients with various degrees of periodontitis to see whether we could separate changes due to inflammation from those associated with gingival overgrowth. This would only have been possible with a much larger group of control and GO patients, stratified based on periodontitis score.

4- Another possible design for this study is a cohort design. With this type of study we can overcome many of confounding variables. Data collection in this study should be done before patients receive CSA. In this case we can record all changes that happen as a side effect of CSA.

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Appendix I:

اینجانب.....فرزند.....متولد سال.....رضایت خود را با انجام عمل جراحی لثه توسط دکتر معصومه نوری به منظور درمان عارضه ناشی از مصرف داروی سایکلوسپورین که برای جلوگیری از رد پیوند کلیه استفاده مینمایم را اعلام میدارم. اینجانب آگاه هستم که در هنگام جراحی نمونه لثه برداشته شده به منظور انجام تحقیقات به کار خواهد رفت.

امضاء

I ..... express my consent regarding periodontal flap surgery by Dr. Masoumeh Nouri for treating my gingival overgrowth. I was been explained that gingival overgrowth is a side effect of cyclosporin usage. I am aware that the discarded gum sample will be used for research purposes.

Signature.....

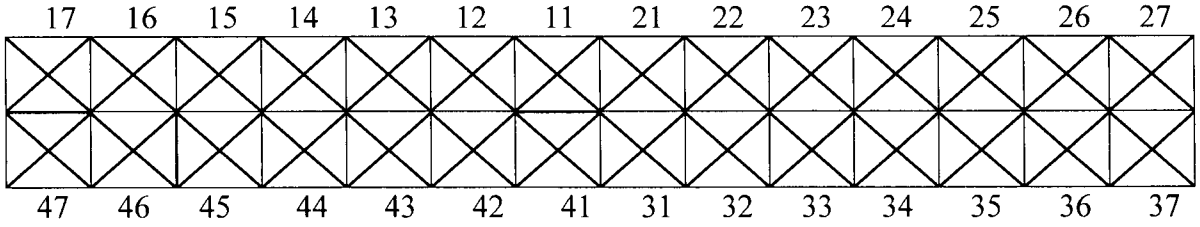
Appendix 2:

- **UNIVERSITY OF BRITISH COLUMBIADepartment of Oral Biological and Medical Sciences**J. B. Macdonald Building, Rm 3982199 Wesbrook MallVancouver BC V6T 1Z3CanadaTel: 1-(604) 822-6819; Fax: 1-(604) 822-3562
- ID NO:..... NAME OF THE CENTER:.....
- PATIENT'S NAME:.....
- PATIENT ADDRESS :.....
- .....
- Date and year of birth.....
- SEX: FEMALE MALE
- Gingival Index: OHI: PD: GOI:
- SMOKING HABIT: YES NO
- IF YES: DURATION: NO OF CIG / DAY
- OTHER SYSTEMIC DISEASES: YES NO
- Explain, using another sheet if necessary
- TYPE OF MEDICATION: NIF CSA PHT DOSAGE:.....
- DURATION:..... Serum level of CSA.....
- Duration of gingival overgrowth (if known)
- The patient has read and understands the institutional consent form
- Signed (by the patient).....
-

Gingival Index or GI (Loe & Silness):

- 0 = Normal gingiva
- 1 = Mild inflammation, slight change in the color, slight edema, no bleeding on palpation
- 2 = Moderate inflammation, redness, edema, and glazing; bleeding on probing
- 3 = Severe inflammation, marked redness and edema, tendency to spontaneous bleeding

The tissues surrounding each tooth are divided into four gingival scoring units: the distofacial papilla, the facial margin, the mesiofacial papilla and the entire lingual margin. Each surface is given one score, the scores are totaled and divided by four; the GI score for each tooth is obtained.



Probing Depth:

- The distance between the margin of the gingiva and the bottom of the pocket in mm. The probing depth is measured for three surfaces (mesial, middle, distal) in facial and lingual surfaces of each tooth.

	17	16	15	14	13	12	11	21	22	23	24	25	26	27
F														
P														
L														
F														
	47	46	45	44	43	42	41	31	32	33	34	35	36	37

Gingival Overgrowth Index or GOI (McGaw et al):

- 0 = No overgrowth, feather-edged gingival margin
- 1 = Blunting of gingival margin
- 2 = Moderate gingival overgrowth, < 1/3 crown length
- 3 = > 1/3 crown length

13	12	11	21	22	23
43	42	41	31	32	33

- **Oral Hygiene effectiveness (OH %):**
- Absence of dental plaque on four surfaces of the Ramfjord teeth

16	21	24
44	41	36