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Institut für Parodontologie und Synoptische Zahnmedizin
der Medizinischen Fakultät der Charité - Universitätsmedizin Berlin

DISSERTATION

The Effects of Enamel Matrix Derivative (EMD) on the Regulation of Connective Tissue Growth factor (CTGF) Expression in Human Osteoblastic Cells via TGF-ß pathway.

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von
Zahnärztin Nora Heng
aus Singapur

Gutachter: 1. Prof. Dr. Dr. Jean-Pierre Bernimoulin
2. Prof. Ph.D. Philip Trackman
3. Priv.-Doz. Dr. Andreas Kage

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For my Beloved Family

Content

Abbreviations

ALP	alkaline phosphatase
AMV	avian myeloblastosis virus
BrdU	5-Bromo-2-deoxy-uridine
BMP	bone morphogenetic factors
BSP	bone sialoprotein
CCN	ctgf/cyr61/nov
CTGF	connective tissue growth factor
ECM	extracellular matrix
ELISA	enzyme-linked immunoassay
EMD	enamel matrix derivative
ERK	extracellular signal-regulated kinases
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
G1/G0	cell resting phase
G2/M phase	chromosomal separation and mitosis phase
GTR	guided tissue regeneration
HERS	Hertwig's epithelial root sheath
iFCS	inactivated fetal calf serum
IGF	insulin like growth factor
IL	interleukin
JNK	c-Jun N-terminal kinases
MAPK	mitogen-activated protein kinases
MMP	matrix metalloproteinase
OC	osteocalcin

OD	optical density
ON	osteonectin
OP	osteopontin
PBS(-)	calcium- and magnesium- free phosphate buffered saline
PDGF	platelet derived growth factor
PDL	periodontal ligament
PI	propidium iodide
RT-PCR	reverse transcriptase polymerase chain reaction
S	DNA synthesis phase
Smads	Signal transducers and transcriptional modulators
TGF-ß	transforming growth factor-ß
TNF-α	tumor necrosis factor-α

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1. Introduction

Periodontal regeneration has been an elusive goal despite the development of widely available regenerative surgical techniques. In recent years, dental tissue engineering has emerged as a new model for periodontal regeneration, with the application of biomaterials that aid in the targeting of proteins, such as growth factors, and regenerative cells to bioengineer the periodontium. Enamel matrix derivative (EMD) is one example for such a biomaterial. Enamel matrix proteins, which are secreted by the Hertwig's epithelial root sheath (HERS), plays an important role in cementogenesis and in the development of periodontal attachment apparatus [1, 2]. A commercial preparation of EMD, EMDOGAIN (Biora Inc.), was introduced in 1997 and is a preparation made from acid extracts of porcine enamel buds. EMD has been increasingly used in promoting periodontal tissue regeneration with promising results. When applied to denuded root surfaces and periodontal bony defects, EMD is found to adsorb onto such surfaces and form an insoluble scaffold complex, which promotes re-colonization of periodontal regenerative cells, inducing periodontal regeneration [3, 4]. Improved clinical outcomes, including significant gain of clinical attachment and reduced probing depth, have been observed following treatment with EMD [5-8].

Although the effects of EMD on periodontal tissue regeneration have been well demonstrated, its mechanisms of action still remain largely unknown. Besides providing a matrix for cell re-colonization, one important question is whether the stimulatory actions of EMD are largely dependent on the effects of growth factors in promoting periodontal regeneration. EMD has been shown to stimulate the production and release of growth factors crucial for periodontal tissue regeneration, such as transforming growth factor (TGF-ß)[9-12]. Recent studies also show the presence of TGF-ß or TGF-ß-like substances in EMD [13-15]. Further studies by KAWASE et al. showed that anti-TGF-ß antibody completely blocked TGF-ß1-induced signaling pathway [13].

Connective tissue growth factor (CTGF) is a member of a recently described ctgf/cyr61/nov (CCN) gene family [16, 17]. It is an extracellular matrix-associated protein that regulates various cellular functions, including fibroblast proliferation, matrix production and survival [18-20]. Studies have established that CTGF expression is strongly related to TGF-ß and is a TGF-ß inducible gene [21-24]. CTGF is proposed to be a downstream mediator of TGF-ß and mediates the cell stimulatory actions of TGF-ß [25, 26]. Studies on CTGF in effect to bone have so far been limited. Recent advances have shown that CTGF is expressed in bone [27-29] and it may play a role in osteoblast development and bone formation [30, 31]. However the mechanism of action of CTGF involved in osteoblastic development is still not well understood.

The interactions between EMD and TGF-ß on osteoblastic cell growth and bone formation have been described but whether EMD has an effect on CTGF expression, which is a downstream mediator of TGF-ß, in osteoblastic cells is still unknown. It is not known whether CTGF, like TGF-ß, plays a role in EMD-induced osteoblastic differentiation.

Our study investigates the effects of EMD on CTGF expression in osteoblastic cells and whether EMD-induced CTGF expression is modulated by TGF-ß signaling pathway. We also examine whether CTGF plays a role in EMD-induced osteoblastic development.

2. Background

2.1. Oral biology structure

The periodontium consists of tooth supporting and investing tissues, which involves root cementum, periodontal ligament, alveolar bone and gingiva. It originates from the dental follicle that is derived from the neural crest. The periodontium consists of a wide array of cell types. Such cells include: periodontal ligament fibroblasts, responsible for ensuring a functional periodontal ligament; osteoblasts and associated progenitor cells, responsible for preserving the surrounding alveolar bone; cementoblasts, root surface lining cells which are involved in cementogenesis[32]. Figure 1 gives an overview of the major events and the actual cells and factors required for formation of the periodontium during development as well as during regeneration.

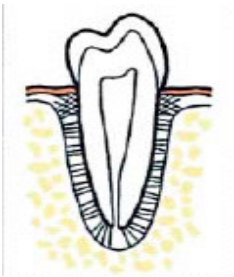
2.1.1. Molecular and cellular biology of alveolar bone

Alveolar bone is a specialized part of the mandibular and maxillary bones and it forms the primary support structure for teeth. The ability of alveolar bone to undergo rapid remodeling is important for positional adaptation of the teeth.

Cellular components comprise osteoblasts, osteocytes and osteoclastic cells. Osteoblasts are the most active secretory cells in bone. They are generally cuboidal or slightly elongated cells that line bone surfaces and are primarily responsible for the production of the organic matrix of bone. Osteoblasts are of mesenchymal origin, containing a cytoplasm rich in synthetic and secretory organelles such as rough endoplasmic reticulum, Golgi apparatus, secretory granules and microtubules. The organic matrix produced by osteoblasts consists predominantly of type I collagen and various other non-collagenous bone proteins, such as osteocalcin (OC), osteonectin (ON), osteopontin (OP) and bone sialoprotein (BSP) [32].

EVENTS

- Migration
- Attachment
- Proliferation
- Matrix synthesis
- mineralisation



TOOTH

EMBRYOGENESIS

PERIODONTAL

REGENERATION

CELLS INVOLVED

Follicle cells	Stem cells
HERS cells	Osteoblasts ,osteoclasts
Stem cells	Cementoblasts,cementoclasts
Osteoblasts	PDL fibroblasts
Cementoblasts	Epithelial cells
PDL fibroblasts	Inflammatory and nerve cells
Odontoblasts	
Inflammatory and nerve cells	

Fig 1: Overview of events and cells occurring during the development and regeneration of the periodontium. (HERS: Hertwig's epithelial root sheath; PDL: periodontal ligament)[33].

The bone matrix is formed from a scaffold of interwoven collagen fibers within and between which carbonated hydroxyapatites ($Ca^{10}[PO^4]^6[OH]^2$) are deposited. Other proteins, including proteoglycans, acidic glycosylated and non-glycosylated proteins, regulate the formation of collagen fibrils and mineral crystals, or provide continuity between matrix components and

between the matrix and cellular components. In addition, small amounts of carbohydrates and lipids contribute to the organic matrix, which comprises approximately one-third of the matrix while the inorganic components account for the remaining two-thirds.

Osteoblastic Differentiation and Bone Formation

Formation of bone involves the proliferation and differentiation of stromal stem cells along an osteogenic pathway that leads to the formation of osteoblasts. There exists a temporal reciprocal relationship between proliferation and sequential development of osteoblast phenotype [34]. First the proliferative period supports DNA synthesis of osteoblast and collagen gene expression. Then down-regulation of proliferation is coupled with increasing differentiation and cells begin expressing collagen as well as alkaline phosphatase (ALP) and OP [35]. ALP and collagen I expression are characteristic of the osteogenic lineage and as the differentiation stage progresses, their synthesis continues to increase [35]. Late osteoblast phenotype markers such as OC and mineralization (calcium) levels increase at later stages of differentiation [36]. Of the many growth and differentiation factors that influence bone formation, bone morphogenetic factors (BMP) and transforming growth factor-ß (TGF-ß) have the most profound effects on bone formation[35, 36, 37]. TGF-ß strongly stimulates expression of matrix proteins by osteoblastic cells [38]. More detailed description of the effects of TGF-ß on osteoblastic cells will be discussed in later chapters. Figure 2 illustrates the events that take place during osteoblast differentiation in normal diploid osteoblastic cells.

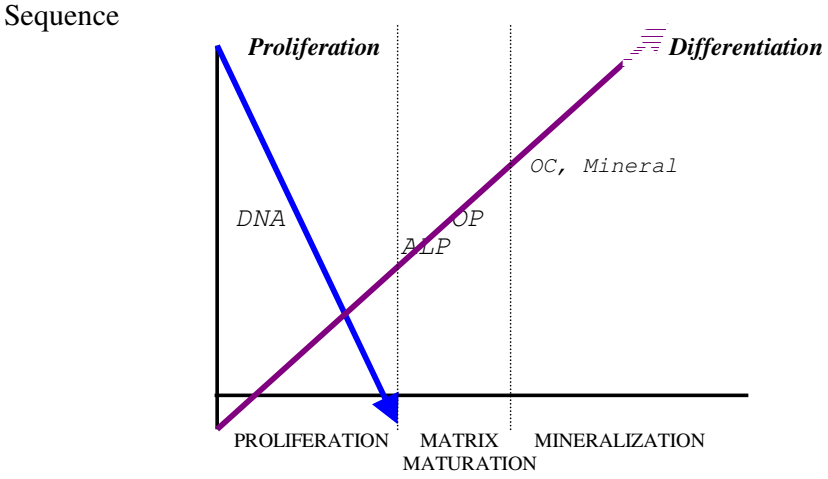


Fig. 2 Relationship between proliferation and osteoblastic maturation. [34]

2.1.2. The roles of Hertwig's epithelial root sheath & enamel matrix proteins

Hertwig's epithelial root sheath (HERS) consists of a double layer of epithelial cells extending apically from the enamel organ. Apical growth of HERS occurs by proliferation of the epithelial cells of the sheath. Continuity between the enamel organ and HERS is lost soon after root formation begins. The apical region of the developing root contains ectomesenchymal progenitor cells that give rise to fibroblasts, pre-odontoblasts and pre-cementoblasts. The role of HERS in root development and cementogenesis has become a focus of considerable attention. Since the epithelial cells of the inner layer of the HERS are analogous to the pre-ameloblasts, it is postulated that they might secrete enamel matrix proteins over the newly deposited root dentin [39-41]. It has been reported that the application of hydrophobic amelogenin peptides to denuded root surfaces promotes new cementum formation [42].

SLAVKIN et al. have reported that HERS secretes polypeptides that are related to, but different from, enamelin and amelogenin proteins [43, 44]. The potential role of these enamel matrix proteins on how they trigger the differentiation of cells capable of forming acellular extrinsic fiber cementum and cellular intrinsic fiber cementum is a primary question that remains mostly unanswered. Yet the concept that epithelial (enamel organ) proteins stimulate cementogenesis has found clinical application in oral tissue regeneration. Further details on enamel matrix proteins are described in Section 2.5.

2.2. Etiology and pathogenesis of periodontitis

Periodontal disease is one of the most common bacterial infections in humans [45]. The inflammatory and degradative processes associated with chronic periodontitis are induced by a critical mass of different pathogens, thereby leading to tissue destruction, possibly by three different pathways [46]:

1. Pathogens directly release proteolytic enzymes that degrade periodontal structures without the intervention of host cells.

2. Pathogens produce products, such as toxins, enzymes, and lipopolysaccharide, that may trigger host cell populations to produce degradative enzymes.

3. Pathogens stimulate an immune response resulting in release of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α.).

The components of the periodontal extra-cellular matrix (ECM), especially collagen, appear to be the main target of degradation in periodontal diseases. Among the host proteases degrading the ECM, matrix metalloproteinases (MMPs) seem to be highly associated with tissue destruction and remodeling events in periodontal diseases [47].

2.3. Periodontal regeneration

The management of periodontal defects, which includes the destruction of the periodontal ligament, cementum and the formation of intrabony defects, has always been a challenge in clinical periodontics. Periodontal regeneration is defined as the restoration of lost periodontium or supporting tissues and includes formation of new alveolar bone, cementum, and periodontal ligament [48].

2.3.1. Basic principles of periodontal regeneration

The regeneration of the periodontal tissues is dependent on four basic components: the appropriate signaling molecules, cells, blood supply and scaffold [49] (Figure 3). Each of these elements plays a fundamental role in the healing process and is interconnected with the generative process of new tissues. Cells provide the machinery for tissue growth and differentiation. Signaling molecules, such as growth factors or morphogens, modulate the cellular activity and provide stimuli to cells to differentiate and produce matrix. New vascular networks promoted by angiogenic signals provide the nutritional base for tissue growth and homeostasis. Finally scaffolds guide and create a template structure to facilitate the above processes crucial for tissue regeneration.

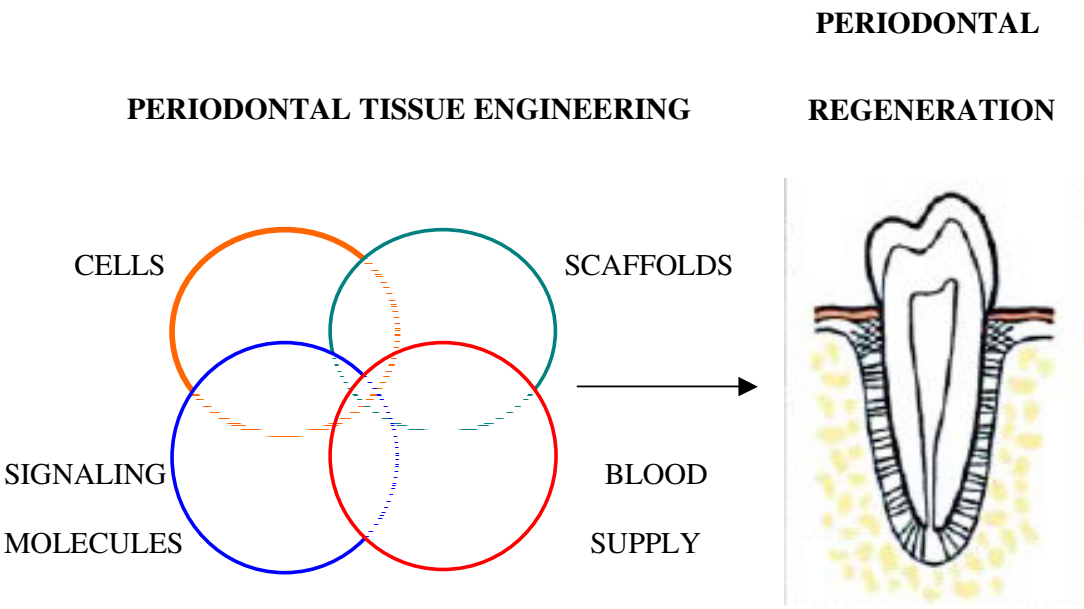


Fig. 3. Critical elements required in periodontal tissue regeneration. Reconstruction of lost periodontal tissue requires the combination of cells, scaffolds, signaling molecules, and a blood supply. [Modified from TABA et al.][49]

2.3.2. Periodontal tissue engineering

Periodontal regeneration has been an elusive goal despite the development of widely available regenerative surgical techniques. In recent years, tissue engineering has emerged as a new model for regeneration for all biomedical fields. Tissue engineering is a relatively new field of reconstructive biology, which utilizes mechanical, cellular, or biologic mediators to facilitate reconstruction or regeneration of a particular tissue [49].

The field of dental tissue engineering is now revealing success in clinical human application. Recent advances in molecular cloning have made unlimited quantities of recombinant growth factors available for application in tissue engineering as an alternative treatment approach for periodontal regeneration [50]. The concept of periodontal tissue engineering had its beginnings with the concept of guided tissue regeneration (GTR).

Guided tissue regeneration (GTR)

The concept of GTR is based on the exclusion of gingival connective tissue cells and prevention of epithelial down-growth into the periodontal defect, thereby allowing cells with regenerative potential, that is, PDL and bone cells, to enter the wound first [51-53]. GTR was based on the presumption that the periodontal ligament contained all of the progenitor cells required for the formation of bone, cementum and periodontal ligament and that selective repopulation of the wound site by the progenitor cells would lead to improved clinical outcomes. However long-term studies and evaluations of this method have indicated that the clinical improvements obtained by this procedure exhibit large variability [50, 54, 55].

Biological factors for periodontal tissue engineering

Recombinant biological factors are used in tissue engineering as an alternative treatment approach for periodontal regeneration. In order to enhance the *in vivo* efficacy, incorporation of bioactive molecules, such as growth factors, into scaffolding materials may facilitate sustained factor release for a period of time. Several growth factors have demonstrated strong effects in promoting periodontal wound repair in preclinical and clinical studies. These bioactive molecules, which include platelet-derived growth factors (PDGF) [56], insulin-like growth factor (IGF-I) [57], fibroblast growth factor (FGF-2) [33], TGF-ß [33], bone morphogenetic proteins (BMPs) [58-60], and enamel matrix derivatives (EMD)[9], have shown positive results in stimulating periodontal regeneration. Table 1 presents a list of growth factors and their effects on PDL cells, cementoblasts (CM) and osteoblasts (OB). The biological properties of TGF-ß and EMD and their applications in an attempt to regenerate periodontal and bone tissues based on the concept of tissue engineering will be further discussed in Sections 2.4 and 2.5.

Table 1: Effects of growth factors on periodontal ligament cells (PDL), cementoblasts (CM) and osteoblasts (OB).

Growth factor effects	Migration			Proliferation			Differentiation			Matrix gene expression		
Cell types	PDL	CM	OB	PDL	CM	OB	PDL	CM	OB	PDL	CM	OB
PDGF	++	?	++	+++	+++	+++	-	-	-	++	+/-	+
IGF-1	++	?	++	+	++	+	-	?	+	+	+/-	++
FGF-2	+++	?	+/-	+++	?	+++	-	?	-	+/-	?	+/-
TGF-ß	+	?	++	++	++	++/-	+	-	+/-	++	+/-	++
BMPs	?	?	++	0	-	+	?	+	+++	?	++	++
EMD	++	?	++	++	++	++	+	+/-	+	+	++/-	++/-

[adapted and modified from Anusaksathien[61]]

Legend

- Inhibitory effect

0 No effect

+ Mild stimulatory effect

++ Modest stimulatory effect

+++ Strong stimulatory effect

? Unknown effect

2.4. Growth Factors

2.4.1. Growth factors: general concepts

Growth factors serve as signaling agents for cells and they function as part of a vast cellular communication network that influences critical functions, such as cell division, matrix synthesis and tissue differentiation. Once a growth factor binds to a target cell receptor, it induces an intracellular signal transduction system that ultimately reaches the nucleus and produces a biological response known as a ligand-receptor interaction. Then the receptor is activated by means of a change in its conformation. Receptors have both extracellular domains that bind to the ligand and intracellular domains that bind to and activate the signal transduction system. Part of this signal transduction system involves a so-called "transcription factor", an intracellular protein that is activated as part of the signaling pathways initiated by the intracellular domain of a receptor. The activated transcription factor travels to the nucleus, binds to the nuclear DNA and induces the expression of a new gene or set of genes. Figure 4 illustrates the mechanism by which growth factors influence cell activity [62].

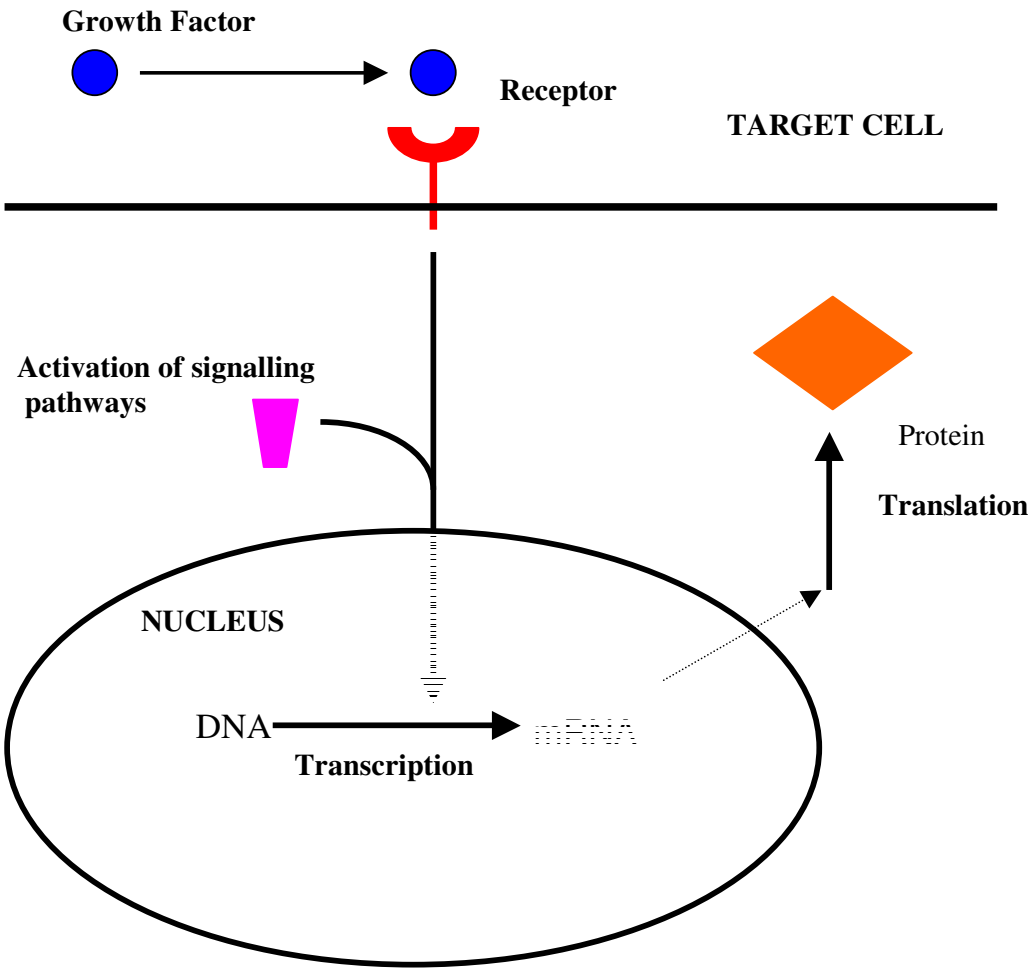


Fig. 4: Diagram demonstrating the mechanism by which growth factors influence cell activity. The ligand binds to the receptor and activates the signal-transduction system. A transcription factor is activated, migrates to the nucleus, binds to the nuclear DNA and induces the expression of a new gene or protein.

2.4.2. TGF-ß and its effects on osteoblasts

Transforming growth factor-ß (TGF-ß) is an ubiquitous peptide that is known to regulate an extensive array of cellular processes, such as proliferation, differentiation, ECM production, angiogenesis, immune responses, and cell death[63] in many cell types including osteoblasts [64, 65]. TGF-ß1, 2 and 3 are members of the TGF-ß superfamily [66, 67]. TGF-ß1, 2 and 3 stimulate mesenchymal cells to proliferate, produce ECM and induce a fibrotic response in various tissues *in vivo*. TGF-ß has been shown to be a strong promoter of ECM production in many cell types,

including PDL fibroblasts [68, 69]. Conversely, TGF-ß1, 2 and 3 inhibit proliferation and induce the apoptosis of epithelial cells. [70].

Bone formation by TGF-ß1 is promoted through chemotactic attraction of osteoblasts, enhancement of osteoblastic proliferation and the early stages of differentiation with the production of ECM proteins, stimulation of collagen expression and proteoglycan synthesis [71]. TGF-ß1 and TGF-ß2 are produced by osteoblasts and incorporated into mineralized bone matrix [72]. TGF-ß is expressed at high levels in mature osteoblasts during bone development and growth [72, 73]. Bone matrix contains significant amounts of latent TGF-ß and very little active TGF-ß [74]. Latent TGF-ß in the bone cell environment is proposed to be activated by proteolytic components of the plasminogen system [75]. Since osteoblasts produce plasminogen activators, these cells can mediate both the production and activation of TGF-ß in the bone cell environment. TGF-ß has been observed to both inhibit and stimulate osteoblastic cell proliferation *in vitro,* depending on TGF-ß concentration, cell density and species and the stage of osteoblastic cell differentiation [65]. Data from many *in vitro* studies have demonstrated the role of TGF-ß1 in every stage of bone formation [76-80].

Despite conflicting results, according to a review by JANSSENS et al [71], most data support the following model: TGF-ß1 increases bone formation *in vitro* mainly by recruiting osteoblastic progenitors and stimulates osteoblastic proliferation, as well as promoting early stages of differentiation, such as bone matrix production [76-78]. However, it blocks later stages of differentiation, such as OC synthesis, and mineralization [79-82].

2.4.3. Connective Tissue Growth Factor (CTGF)

2.4.3.1. Definition

The term “connective tissue growth factor” (CTGF) was first coined by BRADHAM et al. in 1991 [83]. CTGF has emerged as one of six new genes (the others are cyr61, nov, elm1, cop1, and WISP-3) that have been classified into a group of structurally related molecules termed the ctgf/cyr61/nov (CCN) family [16, 17, 84]. The CCN family comprises of both positive (CTGF, Cys61) and negative regulators (nov, elm-1 and cop-1) in cell proliferation, differentiation, embryogenesis and wound healing.

CTGF Gene and Protein Structure

The gene for human CTGF is localised on chromosome 6q23.1 [85]. CTGF has a molecular weight of about 36 to 38 kD and is a cysteine-rich peptide containing 349 amino acids. Figure 5 shows the structural organization of CCN family. Like the other members of the CNN family, CTGF contains binding motifs that are conserved with an IGF-binding domain (module I), a von Willebrand factor type C repeat (module II), a thrombospondin type 1 domain (module III), and a C-terminal domain that contains a cysteine knot (module IV: this module is absent from Cop1)[16]. These modules are proposed to play a role in IGF binding, oligomerization, cell attachment, and dimerization [25]. Various regions within module IV appear to account for much of the heparin-binding ability of CTGF, although a contribution of other domains, such as those in module III [86], may also be involved. As well as potentially regulating its bioavailability, heparin appears to regulate CTGF mitogenic activity [86, 87] and may also function as an adhesion co-receptor.

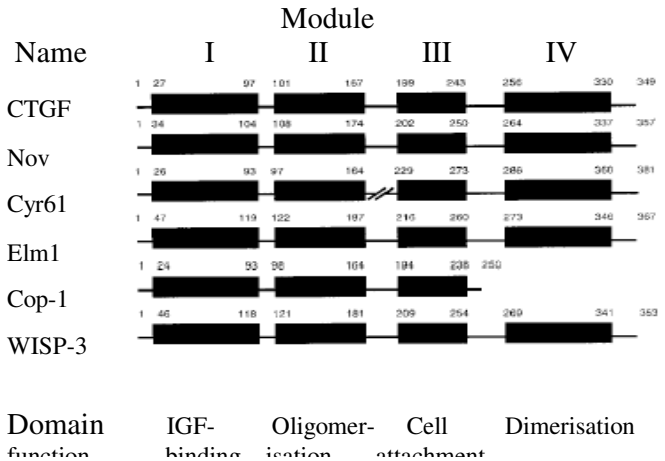


Fig 5. Nomenclature and structural organization of the CCN family.
(adapted and modified from Essam et al.) [25]

2.4.3.2. CTGF biology

CTGF has diverse bioactivities. Depending on the cell types, CTGF has been shown to trigger mitogenesis, chemotaxis, ECM production, apoptosis, and angiogenesis [25]. CTGF was reported to have mitogenic and chemotactic effects on fibroblasts and enhance mRNA expression of collagen I, fibronectin, and ∝-integrin in fibroblasts [87]. Studies have supported a stimulatory role of CTGF in bone formation [27-29]. An overview of the functions of CTGF is shown in Figure 6.

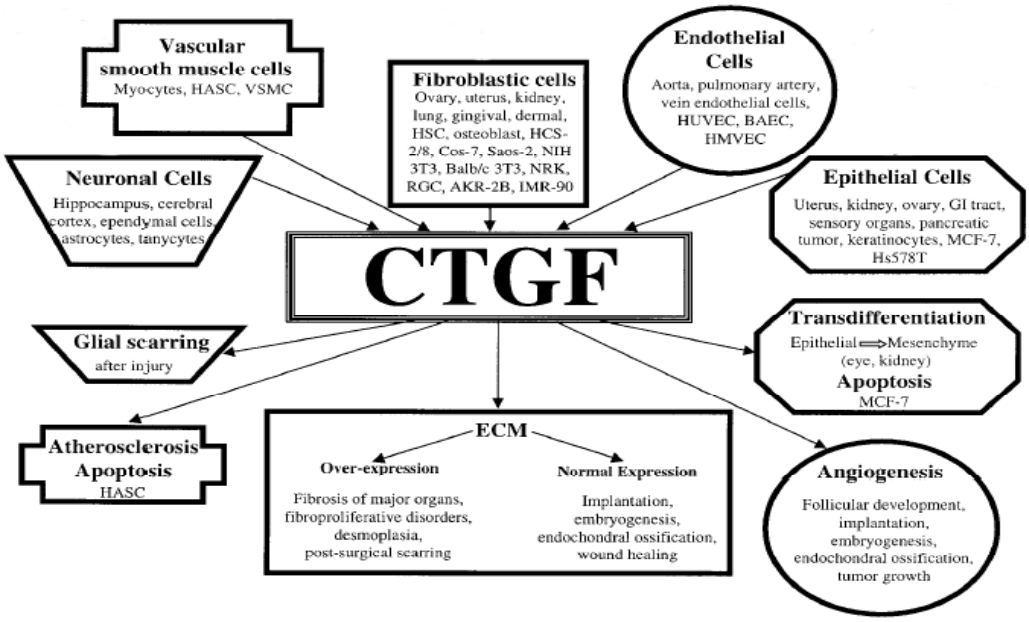


Fig 6. Pathways of production and action of CTGF as a function of cell types. [modified from Essam et al.] [25]

2.4.3.3. Role of CTGF in bone and tooth development

Previous studies have revealed that CTGF stimulates the proliferation and differentiation of osteoblasts [31, 88-90]. In some studies, regulated expression of CTGF in developing tooth germs has been reported [91, 92]. During odontogenesis, CTGF gene expression has been clearly observed not only in the dental mesenchyme but also in the dental epithelium up to the stage of enamel secretion [91, 92]. Therefore, CTGF is now regarded as a common regulator of the development of the tooth- and bone-related structures [92].

2.4.3.4. CTGF-TGF-ß interaction

TGF-ß and CTGF share many functions and it is hypothesized that CTGF is a downstream mediator of TGF-ß. TGF-ß causes a prolonged activation of CTGF gene expression that lasts for 24-36 h after the addition of TGF-ß to the cells [26]. The specificity for regulation of the CTGF gene appears to be controlled at the level of transcription [25, 26].

In vitro studies performed largely on fibroblasts cells have provided a functional basis for the correlation between TGF-ß and CTGF [93-95]. CTGF is expressed in fibroblasts during normal differentiation processes that involve TGF-ß-regulated ECM production and remodeling [93, 94]. Treatment of kidney mesangial cells with TGF-ß was found to result in enhanced levels of CTGF mRNA, as well as increased levels of CTGF protein. Moreover, glucose-induced CTGF expression in these cells was reduced, at least partly, by anti-TGF-ß antibodies [21, 22]. Studies have also shown that CTGF mRNA and protein were produced at high levels in human foreskin fibroblasts after treatment with TGF-ß and protein synthesis inhibitors did not block the induction of CTGF by TGF-ß. [95]. GROTENDORST et al.[23] showed that in fibroblasts, a brief exposure of 1 hour to TGF-ß, in the presence of protein synthesis inhibitors, did not block the amount or duration level of induction of the CTGF gene. In this study it was confirmed that induction of CTGF by TGF-ß was largely at the level of transcription and that other growth factors are only weak or moderate stimulators of CTGF production. The molecular basis for these observations has been attributed largely to a unique TGF-ß response element that is present in the CTGF promoter but absent from the promoters of other CCN genes, as well as other TGF-ß immediate early genes [24]. The data highlights the fact that the transcriptional mechanisms governing CTGF production are distinctive and not shared with other CCN genes. Moreover, they provide a basis for the correlation of CTGF and TGF-ß expression patterns and their shared effects.

Evidence for a functional relationship between the activation of CTGF and TGF-ß has also come from experiments using recombinant CTGF and neutralizing antibodies targeting CTGF, suggesting that CTGF mediates at least some of the effects of TGF-ß on fibroblast proliferation, adhesion, and ECM production, including collagen and fibronectin [24, 26]. The finding that TGF-ß increases CTGF synthesis and that TGF-ß and CTGF share many functions is consistent with the hypothesis that CTGF is a downstream mediator of TGF-ß. For example, TGF-ß-stimulated collagen production was antagonized by anti-CTGF antibodies or antisense oligonucleotides in rat kidney fibroblastic cells and human foreskin fibroblasts [96]. In addition, KOTHAPALLI et al. showed that in rat kidney fibroblastic cells, while CTGF alone did not mimic the effects of TGF-

ß in being able to induce cell proliferation, both CTGF specific antibodies and antisense CTGF were able to inhibit TGF-ß-induced proliferation. This effect was reversed when the fibroblastic cells were co-stimulated with both TGF-ß and CTGF, suggesting the requirement for interactions between both CTGF and TGF-ß-dependent pathways to elicit the cell proliferation [97].

Hence the dogma has arisen that many of the fibrogenic properties of TGF-ß are actually due to its induction of CTGF production and subsequently leads to the stimulation of fibroblast proliferation and ECM production [96]. GROTENDORST et al. have suggested that although CTGF shares some of the biologic functions of TGF-ß, such as stimulating cell proliferation and ECM protein synthesis, it does not share the growth inhibitory function of TGF-ß on epithelial cells [26].

2.4.3.5. Intracellular signaling pathway between CTGF and TGF-ß

There are a few studies regarding the intracellular signaling pathway interlinking TGF-ß and CTGF. Smads are intracellular mediators that modulate the activity of TGF-ß. Studies show that TGF-ß1-induced CTGF expression is mediated through the activation of Smad pathway [63, 98-100]. TGF-ß is known to activate gene expression through the actions of Smad proteins. When TGF is present, Smads 2 and 3 are phosphorylated by the TGF receptor, bind to Smad 4, and migrate into the nucleus to activate expression of TGF-responsive genes, including CTGF gene[71]. The inhibitory Smads 6 and 7 antagonize this pathway of signaling. HOLMES et al. found that transfecting Smads 3 and 4 into fibroblasts enhanced CTGF promoter activity, whereas Smad7 suppressed TGF-induced CTGF expression [101].

Besides the Smad pathway, other pathways, particularly the mitogen-activated protein kinase (MAPK) pathways, are also involved with TGF-ß1-induced CTGF expression. Groups of MAPKs correlated with TGF-ß signaling include extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 isoforms. It is postulated that although the Smad pathway is necessary for CTGF induction, it itself is not sufficient for the activation of target genes; they act synergistically with other signaling cascades and transcription factors to activate gene expression [99]. There are close correlations between Smad and various MAPK cascades. CHEN et al. showed that CTGF induction by TGF-ß1 in cultured mesangial cells was affected by the ras/MEK/ERK signaling cascade. Deletion of a Smad binding site in the CTGF promoter abolished the TGF induction of CTGF [99]. Thus Smads are absolutely required for the TGF induction of CTGF, and MAPK signaling cascades, such as ras/MEK/ERK, can be essential for the TGF induction of genes without directly affecting Smad activity. SATO et al. also

demonstrated that CTGF induction by TGF-ß was mediated through the MAPK p42-p44 pathway [102]. In addition, functional cross-talks between these different pathways may occur [103].

While TGF-ß plays a central role in CTGF biology in several cell types, its action is multifaceted and includes several modes of transcriptional activation of CTGF gene and cooperative interactions with the CTGF protein itself. The existence of TGF-ß independent pathways of CTGF transcription, together with its interactions with diverse binding proteins, highlights the fact that much is yet to be learnt about CTGF production and function.

In summary, studies have established that:

- CTGF expression is strongly related to TGF-ß and is a TGF-ß-inducible immediate or early gene.
- The specificity for regulation of the CTGF gene appears to be primarily controlled at the level of transcription.
- TGF-ß and CTGF share many functions and CTGF is a downstream mediator of TGF-ß.

2.5. Enamel matrix derivative (EMD)

2.5.1. Definition

The involvement of enamel proteins in root formation was first proposed by SLAVKIN and BOYD[104]. It was suggested that the basement membrane contains chemotactic proteins deposited by the Hertwig root sheath (HERS) cells, which serve to direct the migration of pre-cementoblast cells or induce cementoblast differentiation from the dental follicle cells [41, 105]. Several hypotheses have been postulated to explain the function of enamel proteins in root formation: (1) They are involved in the attachment of cementum to root dentine [105]; (2) they initiate cementogenesis [106]; (3) they serve as an inducer of dental follicle cells to differentiate into cementoblasts; studies with monkeys suggested that after the application of enamel matrix proteins to the clean dentin surface of the root, the formation of new acellular cementum was promoted, new alveolar bone was formed and complete attachment of the periodontal ligament was achieved [107, 108]. These possible functions attribute to the hypothesis that enamel proteins could induce periodontium regeneration.

In a study conducted in 1997 by HAMMARSTRÖM et al., it was showed that when porcine enamel matrix was placed in experimental cavities created in monkeys by extracting the incisors, they could initiate the formation of a tissue identical to acellular, extrinsic fiber cementum [42]. Extracted enamel proteins or purified enamel matrix derivative (EMD) resulted in 60–80% formation of new cementum and bone in surgically-produced periodontal defects in monkeys [108]. Studies also show that EMD suspended in propylene glycol alginate (PGA) adsorbs to hydroxyapatite and collagen in the denuded dental roots forming an insoluble spherical complex, which remains on the root surface and promotes re-colonization by periodontal ligament cells [3, 4]. It is therefore suggested that enamel proteins have the ability to promote complete periodontium regeneration by inducing new cementum, periodontal ligament and bone formation.

This product, registered as EMDOGAIN®, has been marketed by BIORA, Inc. It has received FDA approval and is available for the treatment of periodontal defects since 1997. It is derived from a purified acidic extract of developing embryonal enamel from six-month-old piglets, premixed with a propylene glycol ester of alginate (PGA) to improve its viscosity.

2.5.2. Composition of enamel matrix derivatives

The major part of the EMD is composed of the amelogenins, a family of hydrophobic proteins that account for more than 90% of the organic constituent of the enamel matrix[109]. The second largest component of the enamel matrix proteins is the enamelins. The more general term "non-

amelogenin" is now commonly used to describe this high-molecular-weight fraction[108]. It includes proline-rich enamelin[110, 111], tuftelin[112], and ameloblastin (also called sheathlin or amelin)[113]. It also contains serum proteins[114, 115]. Table 2 lists the compositions of EMD.

Table 2: Composition of enamel matrix derivatives

90% Amelogenin	
10% Non-amelogenin:	Tuftelin
	Ameloblastin (sheathlin, amelin)
	Enamelin
	Enamel proteases (eg. MMP-20, EMSP1)
	Serum proteins (eg. Albumin)
	Sulphated enamel proteins

Amelogenins are the most abundant enamel proteins accounting for approximately 90% of all proteins secreted by the ameloblast cells. They are hydrophobic proteins, rich in proline, glutamine, leucine and histidine amino acid residues and exhibit a high degree of sequence conservation [116, 117]. Multiple amelogenin fragments present in the enamel extracellular matrix are the products of alternative splicing of the amelogenin gene and processing of the parent molecules [118, 119]. It is believed that amelogenins function to regulate the orientation, shape and length of the enamel crystals [117, 120]. Mutations in the amelogenin gene are responsible for malformation of the enamel layer in the affected individuals, resulting in hereditary X-linked Amelogenesis Imperfecta.

Tuftelin is an anionic non-amelogenin enamel protein first fully characterized by DEUTSCH et al.[112]. It is expressed as early as the bud stage of tooth development and this protein might serve as a nucleator of de novo crystal formation. The function of this protein in tooth development remains unknown, although recent studies by PAINE et al. suggest that it might function at the level of ameloblast differentiation and/or extracellular matrix secretion [121].

Ameloblastin (amelin and sheathlin) represents 5% of the non-amelogenin mRNAs and has a domain which has been identified in collagen type I as a recognition site for α2ß1integrin [122]. Ameloblastin gene is localized in the region where a family with autosomal dominant Amelogenesis Imperfecta has been linked, suggesting that this protein is important for enamel formation. Ameloblastin is present in the secretory stage of enamel formation. Its localization in

the Tomes' processes of secretory ameloblasts and in the sheath spaces between rod and inter-rod enamel suggests a role in enamel biomineralization.

Enamelin is the largest enamel protein, which concentrates along the secretory face of the ameloblast Tomes' process. It is the parental protein secreted by the ameloblasts and is then processed to produce other low molecular-weight proteins associated with progressive enamel mineralization. It is believed to have a role in enamel biomineralization.

Other factors

Enamel proteases are required for processing secreted amelogenins, ameloblastin and enamelin in the extracellular matrix and subsequently for their degradation and removal from the mineralizing matrix during the maturation stages of amelogenesis. Enamelysin (MMP-20) and enamel matrix serine proteinase-1 (EMSP1) are such enamel proteases. *Sulphated enamel proteins* are present in small amounts [123]. Although their acidic nature suggests that they belong to the family of anionic enamel proteins, their role is unknown.

2.5.3 Role of EMD in periodontal tissue engineering

2.5.3.1 In vitro studies

In vitro studies have demonstrated that EMD affects cellular attachment, mitogenesis, biosynthesis and differentiation. Stimulatory effects are observed in osteoblastic cells, including mouse osteoblast-like OCT-1, MC3T3-E1 and 2T9 cell lines [124, 125]. Exposure to EMD enhanced metabolic activity of osteoblastic cells and promoted biosynthesis of ECM molecules [124, 126]. Numerous investigations have revealed that EMD affects the expression of genes related to mineralization and supports cell differentiation in osteoblastic cells, depending on the specificity and maturity on the cells. Exposure of EMD induced both ALP activity and mineralized bone nodule formation of rat bone marrow stromal cells [127]. EMD down-regulated expression of OC and up-regulated expression of OP in MC3T3-E1 preosteoblasts [128] and osteoblast-like MG-63 cells [126]. SCHWARTZ et al. examined the response of osteoblasts to EMD at 3 stages of osteogenic maturation: proliferation (cell number and [3H]-thymidine incorporation), differentiation (ALP and OC), matrix synthesis (sulphate incorporation and percentage of collagen production). Osteoblastic cell lineages of 2T9 cells (pre-osteoblasts), MG63 human osteoblast-like osteosarcoma cells, and normal human osteoblasts (NHOst cells) were used. EMD was found to affect early stages of osteoblastic maturation by stimulating proliferation but as cells matured in the lineage, EMD enhanced differentiation[126].

Different responses are reported for other cell types. Studies by HAMMARSTRÖM et al. showed that EMD application resulted in more limited epithelial down-growth, aiding periodontal healing[108]. This histologic observation was reinforced by studies done by GRESTRELIUS et al., who found that addition of EMD to cell culture media resulted in enhanced proliferation of PDL cells, as well as increased protein and collagen production and mineralization [4]. EMD induced collagen mRNA expression in murine follicular SVF cells [129]. Production of proteoglycans and extracellular hyaluronan was also promoted by EMD in both PDL and gingival fibroblasts [130]. In contrast, EMD had no significant effect on epithelial cell proliferation *in vitro.* EMD-applied surfaces improved attachment of PDL fibroblasts but had no effect on gingival fibroblasts and epithelial cells, indicating a selective behavior advantageous in the early stages of healing [9, 10]. It may be postulated that the biochemical environment at the root surface following the application of EMD may reduce the epithelial down-growth in a manner similar to the mechanical prevention achieved with the use of barrier membranes in GTR procedures [51-53].

2.5.3.2. In vivo studies

The ability of EMD to regenerate acellular extrinsic fiber cementum was first demonstrated in monkeys [42]. Acellular cementum attached to the dentin was induced 8 weeks later after test cavities were treated with crude porcine enamel matrix. Another study was done by HAMMARSTRÖM et al. with a buccal dehiscence model in monkeys. Regeneration of 60–80% of the cementum defect and new bone formation were obtained by the application of enamel matrix to the denuded root surface in buccal dehiscence model in monkeys [108]. This study has shown that it is possible to induce regeneration of all the periodontal tissues (cementum, periodontal ligament, and alveolar bone) in a way that mimics the normal development of these tissues. The specific characteristics of EMD regarding its bone formation ability (osteoinductive, osteoconductive, or osteogenic) were examined by numerous animal studies [131-133]. Results from these *in vivo* animal studies indicate that EMD has both osteo- and cemento-conductive properties.

Clinical human trials have been conducted to assess the effectiveness of EMD regarding its ability to improve periodontal health. EMD was compared with placebo or with open-flap debridement alone in a split-mouth or parallel-group designs and similar results were found: better outcomes were achieved with EMD treatment in terms of clinical and radiographic findings [134-137]. Some case reports have also presented favorable results showing significant improvement in clinical and radiographic parameters following the use of EMD in the treatment

of intrabony defects [138-140]. Most of the clinical trials and case reports used EMD for the treatment of intrabony defects, since horizontal bone loss defects are not likely to exhibit a successful outcome with regenerative treatment[141]. There are conflicting results regarding the influence of number of defect walls. While several studies reported that the defect configuration affected EMD clinical outcomes significantly [5, 8, 136], other studies did not demonstrate such an effect [7, 139]. 3-walled defects have been associated with greater regenerative potential in both conventional and surgical procedures [6, 136]. However, a comparable success with EMD is also observed in both 1-walled and 2-walled defects [139, 142]. Different mean values for gains in clinical attachment level (CAL) have been demonstrated, ranging from 1.5mm to 6mm [136, 143, 144], and similar results have also been shown for radiographic bone gains [145]. There are comparatively few *in vivo* studies, which examine the effects of EMD in the treatment of furcation defects. JEPSEN et al. and MEYLE et al. both assessed the effectiveness of EMD in the treatment of buccal Class II furcation defects in mandibular molars [146, 147]. They also compared the efficacies of treatment with EMD and bioabsorbable GTR membrane barrier. Both studies reported a significant clinical improvement in EMD-treated cases as compared to untreated control. Horizontal depth reduction was greater in EMD-treated groups than in GTR membrane treatment groups [147].

2.5.4 Mode of action

Although the effects of EMD on periodontal tissue regeneration have been well demonstrated, the mechanisms of its action still remain largely unknown.

EMD as a scaffold for cell attachment

It has been demonstrated that EMD adsorbs both to hydroxyapatite and collagen and to denuded dental roots [3]. It forms insoluble spherical complexes or matrix, and detectable amounts are found to remain at the treated site on the root surface for more than 2 weeks [3, 148, 149]. This appears to be a sufficient period of time to permit recolonization by periodontal ligament cells or undifferentiated cells. In addition, amelogenin has been shown to have a cell-adhesive activity, which may partially explain the therapeutic effects of EMD in periodontal regeneration [150].

EMD modulates bacterial composition and reduces plaque viability

EMD may also promote periodontal regeneration by reducing dental plaque. It was found that EMD had an inhibitory effect on dental plaque viability [151]. The effect of EMD on the growth of

periodontal pathogens was further evaluated *in vitro* [152]. EMD was found to significantly inhibit the growth of the Gram-negative periodontal pathogens whereas the Gram-positive bacteria were unaffected. It is concluded that EMD has a positive effect on the composition of bacterial species in the post-surgical periodontal wound by selectively restricting growth of periopathogens that can hamper wound healing and reduce the outcome of regenerative procedures.

EMD exerts a biological 'guided tissue regeneration' effect

Studies show that EMD have different responses on different cell types. EMD enhances proliferation rate, metabolism and protein synthesis, cellular attachment rate and mineral nodule formation of PDL fibroblastic cells and has a similar influence on cementoblasts and mature osteoblasts [9, 42, 125]. In addition, EMD enhances attachment of these cell types. In contrast to its effects on mesenchymal cells, EMD appears to exhibit a cytostatic action on epithelial cells [4, 9, 153]. These characteristics partly explain the biological 'guided tissue regeneration' effect attributed to EMD by hindering epithelial downgrowth.

EMD as growth factor or stimulates the production of growth factors

Because early studies did not detect the presence of growth factors in EMD preparations[4], it was previously postulated that it acts as a matrix, creating a positive environment for cell proliferation, differentiation, matrix synthesis, and possibly the production of growth factors, which in turn enhance tissue repair and regeneration. Hence this insoluble matrix promotes cells to produce growth factors, including TGF-ß, platelet-derived growth factor (PDGF) and BMPs. LYNGSTADAAS et al. studied various growth factor productions in EMD-cultured human PDL cells. It was found that growth factors (TGF-ß1, IL-6, and PDGF-AB), proliferation, and metabolism of human PDL cells in culture were all significantly increased in the presence of EMD. In contrast, EMD increased cAMP and PDGF-AB secretion in epithelial cell cultures, but inhibited their growth[9]. Results from this and earlier studies further suggest that EMD favors mesenchymal cell growth over growth of epithelial cells. Similarly VAN DER PAUW et al. investigated the effects of EMP on the behavior of human PDL and gingival fibroblastic cells *in vitro*, with special focus on the release of TGF-ß1. It was found that both cell types released significantly higher levels of TGF-ß1 in the presence of EMD[10]. Recent studies showed that expression profiling of human PDL cells stimulated with EMD by cDNA microarray technology revealed that most of up-regulated genes were the ones coded for growth factors and growth factor receptors[11]. Although it has been demonstrated that EMD functions as an insoluble matrix to promote cells to produce growth factors, there is another hypothesis that bioactive molecules released from EMD are also responsible for the tissue regenerative activity of EMD. The

bioactive molecules could be growth factors absorbed to EMD during its preparation or amelogenin peptides. Various amelogenin gene products have been shown to actively participate in cell signaling to stimulate matrix formation and mineralization[154, 155]. These multiple amelogenin gene products exist as a result of alternative splicing. The larger forms are important for enamel mineralization while small amelogenin peptides may have signal transduction function, and have been shown to enhance the expression of collagen, Sox 9 and Cbfa 1 mRNA in vitro. These small amelogenin peptides were able to induce bone formation around implants *in vivo* by enhancing the production of ECM, matrix vascularization and mineralization[154-156]. They have comparable osteogenic activities to recombinant human BMP-2 [154, 155]. The amelogenin peptides also induce the formation of reparative dentin bridge and its functions are comparable to BMP-7 [157]. Protein analysis of EMD revealed the presence of proteolytic enzymes, such as metalloendoproteases and serine proteases, in this commercial preparation[158]. In the tissue repair environment, the presence of proteolytic enzymes can release small amelogenin peptides from EMD, which can subsequently act as soluble growth-like factors to affect neighboring cells. Recent studies have also shown the presence of growth factors in EMD. It has been suggested that EMD have bioactive properties, such as BMP-like activity[159] and TGF-ß-like activity[13].

Hence it is postulated that soluble factors contained in EMD may be responsible for the stimulating effects of EMD. Growth factors, such as TGF-ß and small amelogenin peptides, are potential candidates mediating the effects of EMD.

2.5.5 Evidence of EMD-TGF-ß1 relationship

EMD is found to stimulate the endogenous production of TGF-ß1[9-12]. EMD has also been shown to have TGF-ß1-like functions. KAWASE et al. found the presence of TGF-ß-like molecules in EMD preparations[13]. EMD was subjected to enzyme-immunoassay for TGF-ß1 and it was found that EMD preparations contained TGF-ß1-like immunoreactivity that bound to TGF-ß receptor II. He also showed that significant levels of TGF-ß were present in EMD preparations, which led to rapid phosphorylation of the MAP kinase family and translocation of Smad2 into the nucleus in both oral epithelial and fibroblastic cells. Further study by them showed that anti-TGF-ß antibody completely blocked TGF-ß1-induced Smad2 pathway[153]. SUZUKI et al. fractionated EMD and found that TGF-ß-like activity was detected in low molecular weight fractions[14]. HE et al. concluded that direct contact is not required for EMD-induced cell proliferation and soluble

factors such as TGF-ß1 and small amelogenin peptides may be factors mediating the effects of EMD[15]. Therefore, TGF-ß1 remains to be a candidate mediating the effects of EMD.

3 Aims of study

Enamel matrix proteins (EMD) have been shown to stimulate the production and release of growth factors crucial for periodontal tissue regeneration, such as TGF-ß[13, 14]. CTGF is a downstream mediator of TGF-ß and mediates the cell growth stimulatory actions of TGF-ß[25, 26]. The interactions between EMD and TGF-ß on osteoblastic cell growth and bone formation have been described but whether EMD is affecting CTGF expression in osteoblastic cells is still unknown. As CTGF is proposed to mediate TGF-ß actions, it is not known whether CTGF, like TGF-ß, plays a role in EMD-induced osteoblast differentiation.

The aims of our study were to examine the effects of EMD in human osteoblastic cells on CTGF expression and whether EMD-induced CTGF expression is modulated by TGF-ß signaling pathway. We also examined the role of CTGF in cell cycle progression, cell proliferation and matrix mineralization of osteoblastic cells treated with EMD. The aims were achieved by the following approaches using:

- semi-quantitative RT-PCR to assess CTGF mRNA expression
- Western blot to assess CTGF protein expression
- BrdU ELISA to examine the effect of CTGF in EMD-induced DNA synthesis
- Flow cytometry to assess cell cycle kinetics.
- Alizarin red staining to assess matrix mineralization.

4 Methods and Materials

4.1. Cell culture

Saos-2 osteoblastic cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). The osteoblastic cell line was originally derived from the primary osteogenic sarcoma of an 11-year-old Caucasian woman.

The vial containing the cells was frozen with 70% McCoy's medium, 20% inactivated fetal calf serum (FCS), 10% dimethyl sulfoxide (DMSO) at 2 x 10^6 cells/ ampoule and stored in liquid nitrogen phase. For cell culturing process, the vial was quickly transported from liquid nitrogen and immediately thawed. To remove the cryoprotective agent, 10ml warm complete medium (refer to * below) was slowly added and spun at 1000g for 10 minutes. The medium was then decanted and pellet was resuspended in a 75cm^2 flask containing 10ml of complete media. The complete culturing media consisted of 85% McCoy's 5A modified medium containing 2.2g/l $NaHCO_3$ (Biochrom AG, Berlin, Germany), 15% inactivated FCS (iFCS) (Biochrom AG, Berlin, Germany) and 7.5µl/ml L-Glutamine (Biochrom AG, Berlin, Germany)*. Cells were incubated at 37^oC and 5%CO_2 in air atmosphere. On the following day, cells were checked visually for adherence and viability and the medium was changed. Thereafter the medium was changed every 3 days. When the cells reached confluency, they were rinsed with phosphate-buffered saline without calcium and magnesium [PBS(-)] (Biochrom AG, Berlin, Germany) twice and trypsinized with 3ml of Trypsin/EDTA (0.05%/0.02% in PBS)(Biochrom AG, Berlin, Germany) until the cell layer was dispersed. Cells were further subcultured in a ratio of 1:3 or cultured into dishes or microwells for the experimental applications. All experiments were performed using cell cultures between the 3rd and 7th passages.

4.2 Preparation of test reagent (EMD)

A syringe containing 0.7ml of sterile enamel matrix derivatives (EMD) (30mg/ml) (BIORA,Malmö, Sweden) was placed in a 37^oC water bath to reduce its viscosity. The contents were then dissolved in sterile Hank's solution without calcium and magnesium to yield a 1mg/ml stock solution. The solution was stored in the refrigerator for no longer than 3 weeks.

4.3 RT-PCR

Cell treatment

Cells were trypsinized and subcultured in 6-well plates (Nunc, Roskilde, Denmark) at the density of $2x10^5$ cells per dish in McCoy's media supplemented with 15% inactivated FCS. After reaching confluency, cells were serum-starved for 24h prior to treatment.

Time-dependent experiment was first done whereby cells were treated with and without 100µg/ml EMD in serum-free McCoy's media for 6, 12 and 24 hours prior to mRNA extraction. Concentration-dependent experiments were also done whereby cells were cultured in serum-free McCoy's media with EMD concentrations of 0 to 100µg/ml for 12 hours (as determined by previous time-dependent experiment).

To assess the effects of EMD and anti-TGF antibody on CTGF mRNA expression, cells were treated for 12 hours in serum-free McCoy's media, media containing 100µg/ml EMD and media containing 5ng/ml human TGF-ß1 (Austral Biologicals, California, USA); all 3 groups are with and without 5µg/ml monoclonal anti-TGF-ß1,-ß2,-ß3 antibody (R&D Systems, Minneapolis, US).

Isolation and quantification of mRNA

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen, West Sussex, UK). In brief, the monolayer cell cultures were first rinsed with 5ml/well PBS (Biochrom AG) and were then lysed and scrapped with 200µl/well lysis buffer mixture (1ml RLT-buffer, Qiagen; 10µl ß-Mercaptoethanol). The cell samples were pooled and the concentration of RNA in each sample was determined by measuring the absorbance at 260nm (A_{260}) in a spectrophotometer (Uvikon 922, Bio-Tek Kontron).

Reverse transcriptase (RT)

RNA samples were diluted to a concentration of 1 µg/ 11.5 µl in 0.5 ml aliquot prior to RT. The procedure was done on ice. To remove secondary structure, the RNA extracts was heated for 5 min at 70°C in the Thermocycler (TGradient, Whatman Biometra, Göttingen). For cDNA synthesis, 8.5µl of RT reaction mixture (Table 3) was added. The reaction mixtures were incubated at 42°C for 1 h. Avian myeloblastosis virus (AMV) reverse transcriptase was inactivated by heating the reaction mixtures at 95°C for 5 min. 80µl DNAse- and RNAse-free water (Roth, Karlsruhe) was added to each sample to make up a total volume of 100µl before

storing at -20°C. To test for DNA contamination of the RNA extracts, control reaction mixtures were prepared as described above, but AMV reverse transcriptase was not added.

Table 3: RT reaction mixture (added per aliquot)

	µl
AMV reverse transcriptase (Promega)	0.75
deoxynucleotide triphosphates (dNTP) (10mM) (Promega)	2
Hexamer primer (0.2µg/µl)(Roche)	1
RNAsin® (Promega)	0.75
AMV Reverse Transcriptase 5X Reaction Buffer (Promega)	4
Total	8.5µl

Primer design for PCR

The primers for the target genes were designed with computer assistance using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge,MA) as shown in Table 4. Glyceraldehyde-3-phosphate (GAPDH) mRNA serves as a housekeeping gene to ensure equivalent DNA loading. Primers were synthesised by TIB®MOLBIOL, Berlin.

Table 4

Primer name	Primer Sequences
CTGF	
Forward	GGCCTCTTCTGTGACTTCGG
Reverse	TGCAGGAGGCGTTGTCATT
GAPDH	
Forward	ACCCAGAAGACTGTGGATGG
Reverse	TGTGAGGGAGATGCTCAGTG

Qualitative PCR

For amplification of cDNA, 5 µl of the RT reaction product was added to 20µl of Mastermix (Table 5) with primers as described above. 32 cycles and 26 cycles were used for the amplification of CTGF and GADPH cDNA, respectively. The reaction conditions for CTGF and GAPDH are shown in Table 6.

Table 5: Mastermix (per probe)

Ammonium-PCR-Buffer 10x (Sigma)	2.5µl
deoxynucleotide triphosphates (dNTP) (10mM)(Promega)	0.5µl
Primer (0.5µg/µl) (TIB®MOLBIOL)	
Forward	0.25µl
Reverse	0.25µl
RedTaq-Polymerase (1U/µl) (Sigma)	1.25µl
DNAse and RNAse free water (Roth)	15.25µl
Total volume (per probe)	20.00µl

Table 6: PCR reaction conditions for CTGF and GAPDH cDNA

Initial denaturation at 94°C for 3 min	
Annealing for 1 min	
Annealing temperature for CTGF is 65°C	CTGF: 32cycles
Annealing temperature for GADPH is 60°C	GADPH: 26 cycles
Elongation at 72°C for 1 min	
Terminal elongation at 72°C for 5 min	
Pause at 4°C	

Gel electrophoresis

1.5% agarose gel was prepared by completely dissolving 1.5g of agarose (Promega EEO 0.06-0.13, Madison, USA) to 100 ml of Tris-Acetate-EDTA buffer (TAE). The agarose was cooled to about 55°C before adding 4µl of ethidium bromide and swirled gently. It was poured into a gel tray and a gel comb was placed into the slots at the top of the gel. It was left to solidify for 20mins. After solidification, it was then placed into a TBE-containing electrophoresis chamber (Biorad, München, Germany). The comb was gently removed and 10µl of cDNA samples were placed in each slot. The gel underwent electrophoresis at 100V for 45min. Reaction products were visualized with ethidium bromide DNA staining under UV light. Fragment sizes (CTGF:0.45 kb) were confirmed by comparison with a 1-kb DNA ladder molecular weight marker (Life Technologies, Maryland, USA). The densitometry of the bands on agarose gels resembling the RT-PCR products were quantified with the ArgusX1V.2 system and the volume tool of the QuantityOne software (Bio-Rad, CA, USA.).

4.4. Western Blotting

Cell treatment

Cells were trypsinized and subcultured in 60mm diameter dishes (Nunc, Roskilde, Denmark) at the density of $1.4x10^6$ cells per dish in McCoy's media supplemented with 15% inactivated FCS. After reaching confluency, cells were serum-starved for 24h prior to treatment.

Time-dependent experiment was first done whereby cells were treated with and without 100µg/ml EMD in serum-free McCoy's media for 12, 24, 48 and 72h prior to protein extraction. Concentration-dependent experiments were also done with EMD (0, 25, 50 and 100µg/ml) for 12 hours. Similarly, to study the effects of EMD and anti-TGF antibody on CTGF mRNA expression, cells were treated with experimental medium consisting of serum-free McCoy's media or serum-free McCoy's media containing 100µg/ml EMD or serum-free McCoy's media containing 5ng/ml human TGF-ß. The 3 treatment groups were treated with and without 5µg/ml of anti-TGF-ß antibody for 48h as determined by previous time-dependent experiment.

Protein extraction

Upon reaching near-confluence, cells were then washed twice in chilled PBS(-). Cells were harvested on ice by scraping with cold lysis buffer containing 1X Protease Inhibitor Cocktail (Table 7). Protein concentration was determined using Bradford method[160] with a Bio-Rad protein assay reagent. Protein samples were resuspended in Laemmli buffer (Biorad Laboratories, USA) as described by Laemmli [161] and boiled for 5 min. Protein extract (80µg/lane) was fractionated by sodium dodecyl sulfate- 12.5% polyacrylamide electrophoresis gel (SDS-PAGE) and then blotted onto Hybond-ECL membrane (Amersham, Dreieich, Germany). The membranes were blocked with Odyssey blocking buffer (LICOR Inc., Bad Hamburg, Germany) for 1h at room temperature and then incubated overnight at 4^oC with blocking buffer containing rabbit polyclonal CTGF antibody (anti-CTGF-ab6992, Abcam, Cambridge, UK) and mouse monoclonal ERK2 antibody (sc1647, Santa Cruz Biotechnologies, CA, USA), at a dilution of 1:2000 each. Anti-ERK2 antibody was used to confirm equal protein load. The next day, the membranes were washed thoroughly with washing buffer containing PBS(-) and 0.1% Tween 20. It was then incubated for 1h at room temperature with blocking buffer containing fluorochrome-conjugated secondary antibodies: CY5.5 conjugated anti-mouse IgG and IRDye™ 800 conjugated anti-rabbit IgG (Rockland Immunochemicals, PA, USA) at a dilution of 1:2000 each. Proteins were visualized by using an Odyssey infrared imaging system (LICOR Inc., Bad Hamburg, Germany). Protein sizes (CTGF: 38kDa, ERK-2: 44kDa) were

confirmed by comparison with a protein molecular weight marker (Bio-Rad's SDS-PAGE Standards). Relevant band intensities were quantified by densitometric analysis using software from Odyssey imaging system (LICOR Inc.). Figure 7 describes in greater detail the protocol of Western blotting for Saos-2 cells.

Table 7: Preparation of Lysis Buffer (1ml)

PBS without calcium and magnesium	833µl	
Tris-HCL 50mM,pH7.4	100µl	from 500mM stock
EDTA 0.25mM	1µl	from 250mM stock
PMSF 1mM	10µl	from 100mM stock
Antipain, 10µg/ml	5µl	from 5mg/ml stock
Leupeptin 10µg/ml	5µl	from 5mg/ml stock
Pepstatin A 10µg/ml	5µl	from 5mg/ml stock
Triton-X 100 1%	50µl	from 20% stock

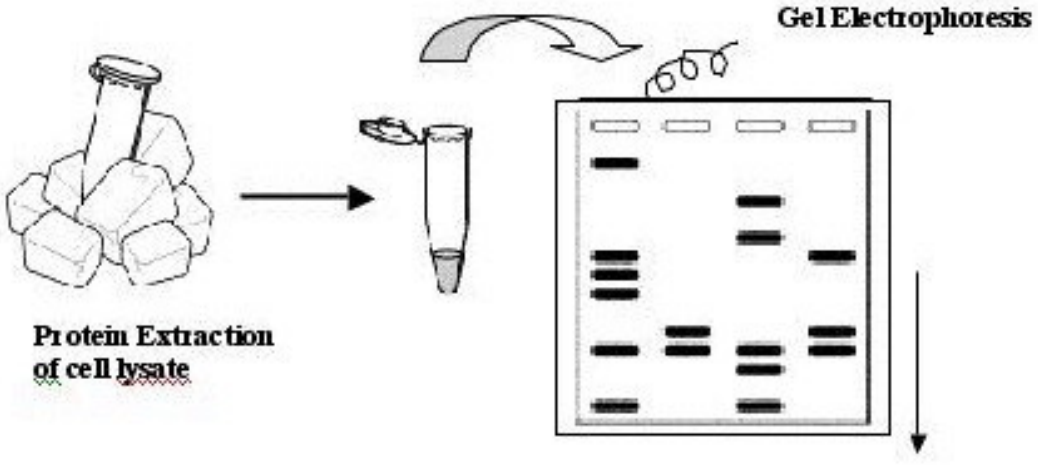
Fig 7 Protocol of CTGF protein analysis by Western blotting

Protein extraction of cell lysate

- Saos cells were washed and harvested on ice by scraping with 50µl/dish of lysis buffer.
- The cell lysate was centrifuged at 14000rpm for 10 minutes at 4°C. The supernatant was collected and frozen at this point for long-term storage at -80°C.
- The protein concentration was determined by Bradford assay.
- The protein samples were mixed with Laemmli buffer and stained with 2-mercaptoethanol.

Gel electrophoresis

- 1-mm-thick gels was prepared with 12.5% resolving gel (based on 30:1 acrylamide/bisacrylamide), using BioRad™ Mini-PROTEAN 3 System
- 80µg of protein extracts were loaded onto the gel.
- Electrophoresis was performed for about 1.5 h at 100V, till the bands reached the base of the gel.

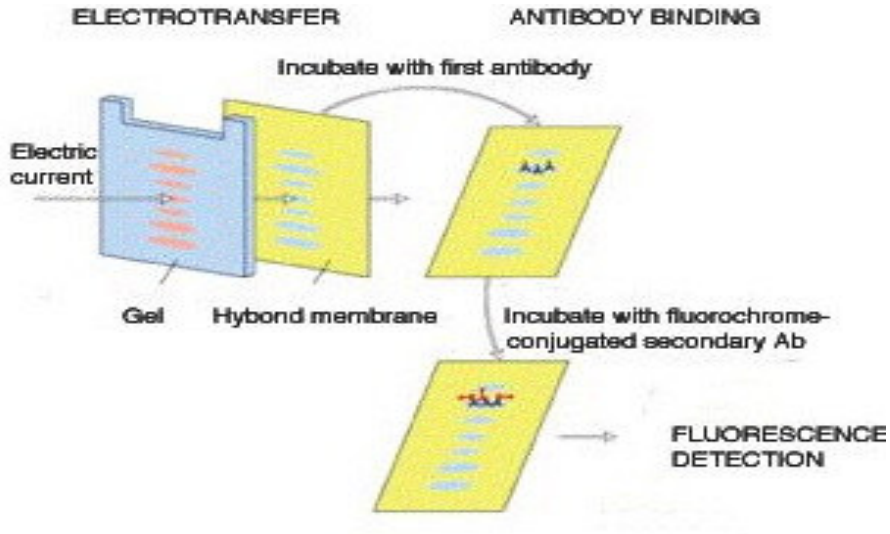


Western blotting

- Following electrophoresis, the gel was blotted onto a piece of Hybond™ ECL blot for 1h at 100V, 350mA.
- The blot was incubated in Odyssey blocking buffer for 1 h at room temperature with gentle shaking.
- It was then incubated overnight at 4^{o}C with blocking buffer containing rabbit polyclonal CTGF antibody and mouse anti-ERK antibody at a dilution of 1:2000 each.
- The blot was washed: three 5-min in 0.1%Tween20-containing PBS(-), one 5-min wash in PBS(-).
- The blot was incubated in blocking buffer containing fluorochrome-conjugated secondary antibodies solution for 1h at room temperature with gentle shaking.
- The blot was washed as described in previous step.

Fluorescence imaging

- Protein sizes (CTGF:38kDa, ERK-2: 44kDa) were visualized by using an Odyssey infrared imaging system in different fluorescence channels (700 and 800 nm).
- Band intensities were quantified by densitometric analysis using software from Odyssey imaging system.



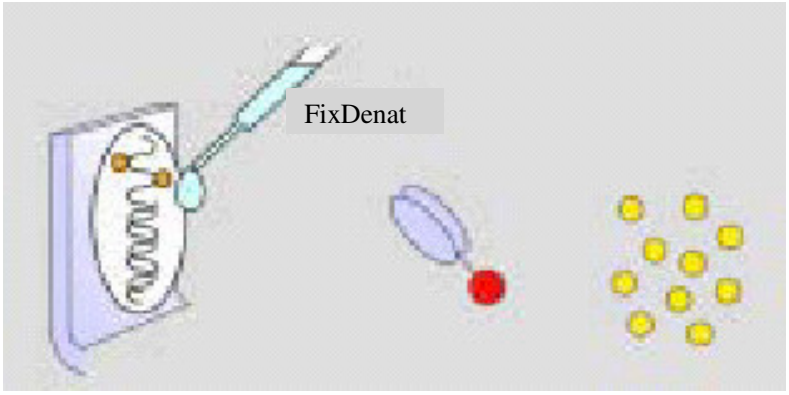
Schematic diagram on steps of Western blotting after gel electrophoresis.
(modified from http://www.boneslab.bio.ntnu.no/ BI211/Western.jpg)

4.5. BrdU assay

4.5.1. Principle of BrdU assay

The colorimetric cell proliferation ELISA (BrdU cell proliferation kit, Roche, Basel, Switzerland) measures cell proliferation by quantitating 5-bromo-2´-deoxy-uridine (BrdU) incorporated into the newly synthesized DNA of replicating cells. The technique was first described by Gratzner[162]. It offers a non-radioactive alternative to the [^{3}H]-thymidine-based cell proliferation assay with comparable sensitivity[163]. The assay is a cellular immunoassay, which uses a mouse monoclonal antibody to direct against BrdU.

Test principle of colorimetric BrdU assay (according to Roche)



Fixed cells with partially denatured BrdU-labeled DNA	anti-BrdU-PO Fab-fragment	TMB substrate

4.5.2. Assessment of DNA synthesis in Saos2-cells by BrdU

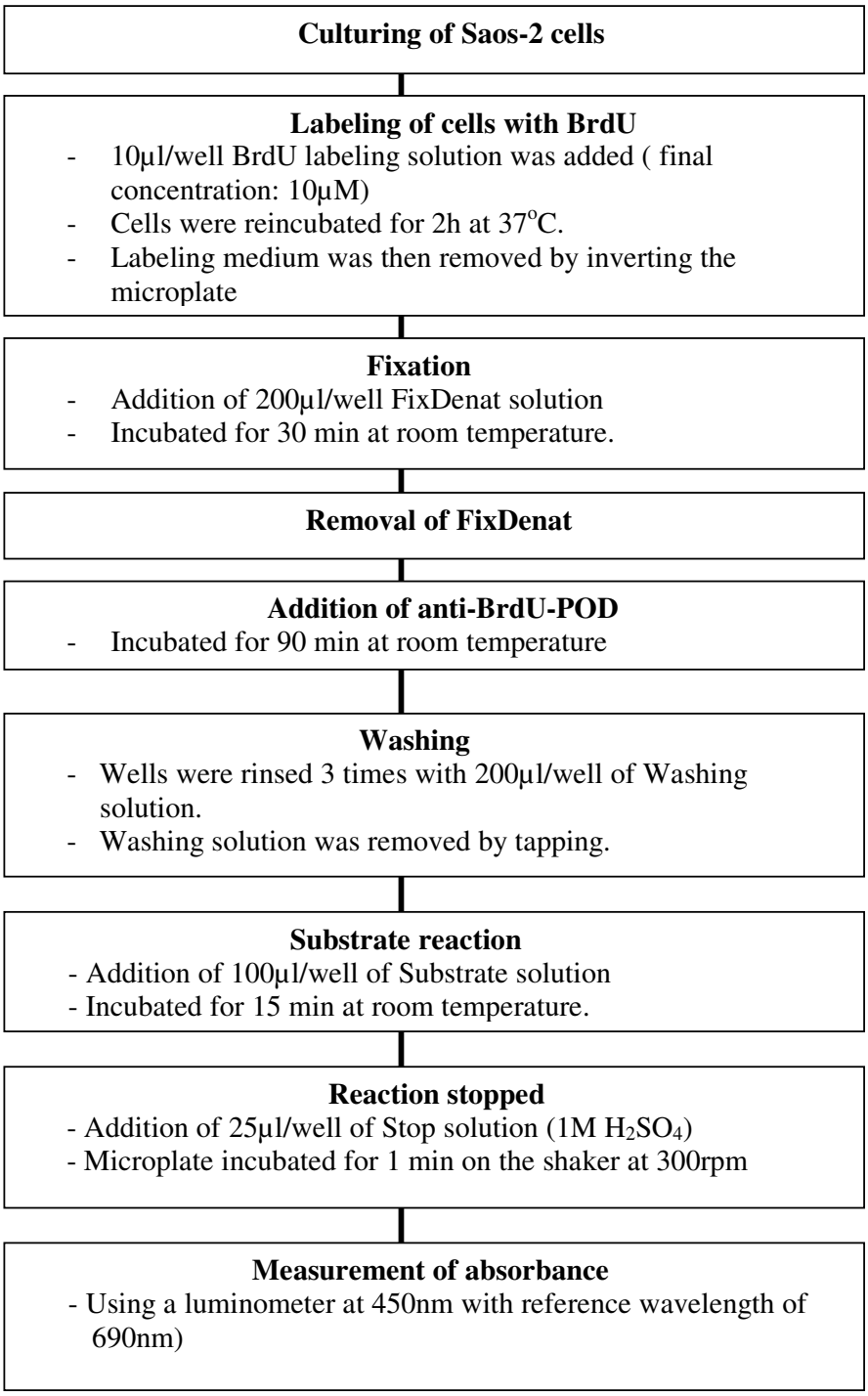
Cell treatment

SAOS cells were trypsinized and seeded in 96-well dishes (Nunc, Denmark) at the density of $5x10^3$ cells/well ($5x10^4$ cells/ml) at 37^{o}C for 48 hours. Experimental medium consisted of McCoy's media containing 2% iFCS supplemented with 100µg/ml EMD or 5ng/ml human TGF-ß, with and without 500ng/ml anti-CTGF antibody (Abcam, Cambridge, UK) as suggested in the literature [164-166]. Concentration-dependent experiments were also done whereby cells were cultured in 2% iFCS-containing McCoy's media with various concentrations of EMD between 0 and 100 µg/ml, with and without anti-CTGF antibody.

Analysis of DNA synthesis

Nucleic acid synthesis was assessed by BrdU colorimetric immunoassay (Roche, Switzerland) in accordance with manufacturer's protocol. The assay is based on measuring BrdU incorporation (2 hours labeling time) in place of thymidine into newly synthesized DNA of replicating cells by ELISA. Optical densities (OD) were measured using Bio-Rad microplate reader 3550 at 405nm and quantified using Microplate Manager ® 4.0 software from Bio-Rad (California, USA). The presented results are based on 4 independent experiments (each having similar results) with 6 parallel cultures each. Results are given as percentage values, related to the OD of untreated control cultures. Table 8 describes the procedure in greater detail.

Table 8: Protocol of BrdU assay procedure (according to Roche)



4.8. Flow cytometry (FACS)

4.6.1. Principle of FACS

Flow cytometry or FACS is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light.

It measures a particle's or cell's:

1. relative size
2. granularity or internal complexity
3. relative fluorescence intensity.

A flow cytometer is made up of three systems: fluidics, optics, and electronics (Figure 8).

• The fluidics system transports particles in a stream to a laser beam.

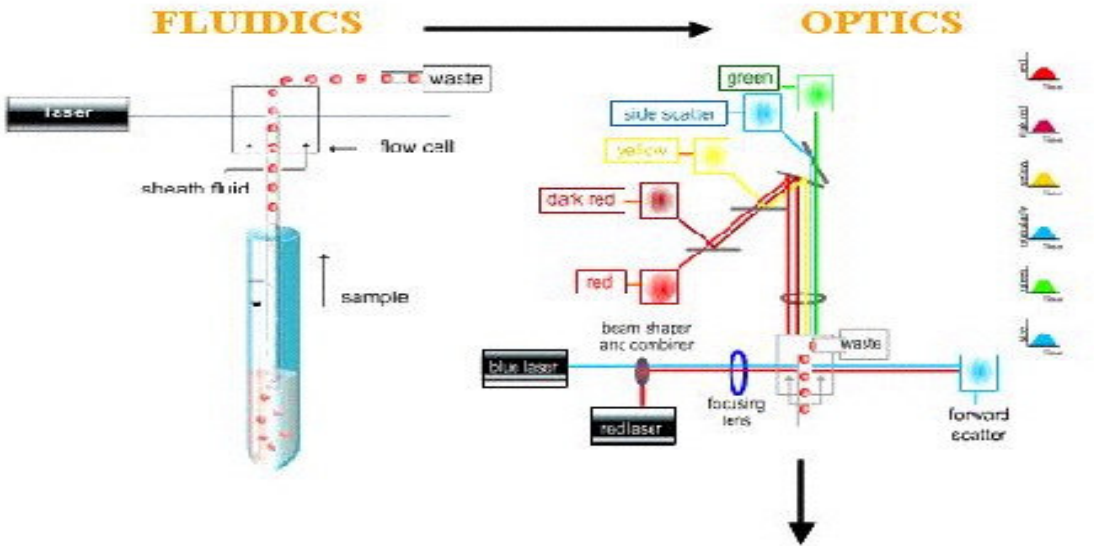
• The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to appropriate detectors.

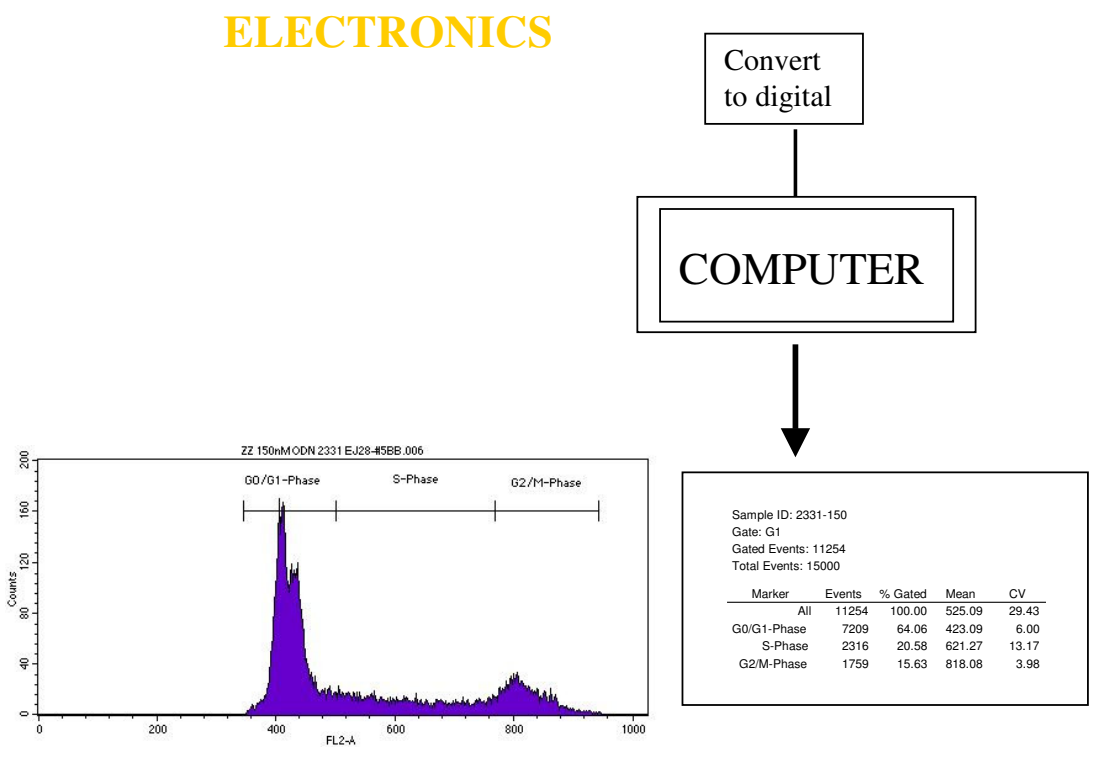
• The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

Particles are carried to the laser intercept in a fluid stream. When particles pass through the laser intercept, they scatter laser light. Appropriately positioned lenses collect the scattered and fluorescent light. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them.The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. Three types of data can be generated:

Forward scatter (FSc)	Approximate cell size
Side or orthogonal scatter (SSc)	Cell complexity or granularity
Fluorescent	Fluorescent labeling is used to investigate cell structure and function.

Fig 8: Schematic overview on the principle of FACS (modified from BD Biosciences).



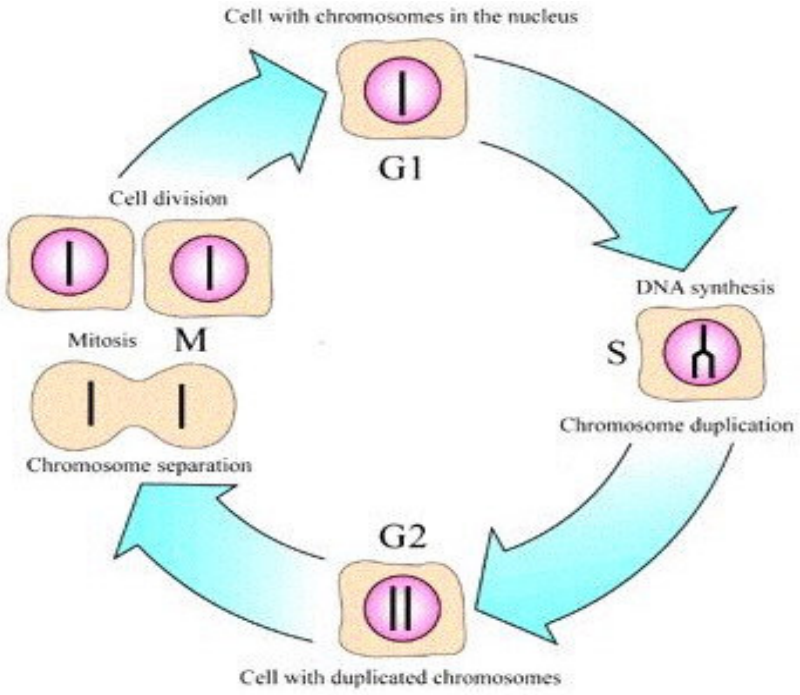


The phases of the cell cycle

The cell cycle consists of several phases (Figure 9). In the first phase (G1) the cell grows and becomes larger. When it has reached a certain size it enters the next phase (S), in which DNA-synthesis takes place. The cell duplicates its hereditary material (DNA-replication) and a copy of each chromosome is formed. During the next phase (G2) the cell checks that DNA-replication is completed and prepares for cell division. The chromosomes are separated (mitosis, M) and the cell divides into two daughter cells. Through this mechanism the daughter cells receive identical chromosome set ups. After division, the cells are back in G1 and the cell cycle is completed.

Cells in the first cell cycle phase (G1) do not always continue through the cycle. Instead they can exit from the cell cycle and enter a resting stage (G0).

Fig 9: Phases of cell cycle



Cell cycle analysis

The measurement of the DNA content of cells is one of the first major applications of flow cytometry. The DNA content of the cell can provide a great deal of information about the cell cycle, and consequently the effect on the cell cycle of added stimuli.

Propidium iodide (PI) binds to DNA and becomes fluorescent. DNA staining can then be used to study the cell division cycle. The DNA content can be inferred from the extent of fluorescence of a DNA-binding PI. Relative DNA content shows the proportion of cells in G1, G2 and S phases. Stained cells with one copy of their genetic material (a haploid cell) will be half as bright as cells with two copies (a diploid cell). A cell varies between these states during the cell cycle and FACS is able to determine its position in the cell cycle based on its DNA content.

4.6.2. Cell cycle analysis of Saos-2 cells

Cell treatment

SAOS cells were trypsinized and seeded in 6-well plates (Nunc, Denmark) at the density of $3x10^5$ cells/dish at 37^{o}C for 48h in 2% iFCS-containing medium. As previously described in cell stimulation for BrdU, similar experimental media were prepared which consisted of McCoy's media containing 2% iFCS (control) or McCoy's media containing 2% iFCS and 100µg/ml EMD or McCoy's media containing 2% iFCS and 5ng/ml human TGF-ß1 (Austral Biologicals, California, USA), with and without 500ng/ml anti-CTGF antibody (Abcam, Cambridge, UK). Concentration-dependent experiments were also done whereby cells were cultured in 2% iFCS-containing McCoy's media with various concentrations of EMD between 0 and 100 µg/ml, with and without anti-CTGF antibody. After the 48h treatment, cells were washed with PBS(-) and trypsinised with 200µl of Trypsin/EDTA per dish until the cell layer was dispersed. The trypsin was neutralized with McCoy's media containing 2% serum. Cells were counted using a hemocytometer and approximately $2x10^6$ cells per each group were collected in eppendorfs and centrifuged at 1000rpm for 5 min at 4^{o}C. The supernatant was discarded and the cells were further washed 3 times with 1ml of cold PBS(-) supplemented with 2% serum. The cells were then fixed in 70% ethanol at 4°C for 30 minutes. The fixed cells were centrifuged and washed twice with 1 ml of PBS(-). They were treated with 600µl of cold PBS(-) containing 2% serum, 0.5mg/ml RNase A (Sigma) and stained with 50µg/ml Propidium Iodide (Sigma). After incubation at room temperature for 10-30 minutes, cell samples were placed in 12 X 75mm Falcon tubes and analyzed by flow cytometry BD FACSCalibur^{TM} system (BD Biosciences, San Jose, USA). Flow cytometric analysis was carried out at a flow rate of 1,000 events/s and a total

of 10,000 events were counted. Data obtained were analysed using CellQuest™ Pro (BD Biosciences).

4.7. **Alizarin Red Staining**

Cells were subcultured in 6-well plates (Nunc, Denmark) at the density of $2x10^5$ cells per well. Upon reaching confluency, cells were serum starved for 24h prior to treatment. Experimental medium consisted of McCoy's media containing 2% iFCS and 7.5 µl/ml L-Glutamine, supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2.5 µl/ml amphotericin B and 50 µl/ml ascorbic acid (all supplied by Sigma-Aldrich, Hamburg, Germany) . Similar to cell treatment in BrdU assay and cell cycle analysis, the cultures were stimulated with 100 µg/ml EMD or 5 ng/ml human TGF-ß, with and without anti-CTGF antibody. From day 8 of culture, 5 mmol/l ß-glycerophosphate was added in order to induce mineralization.

To determine the degree of mineralization, the osteoblastic cultures were stained with alizarin red S after 21 days of stimulation. After washing with PBS, the cells were fixed with 70% ethanol for 10 min, and then stained with 0.5% alizarin red S in H_2O, pH 4.0, for 15 min at room temperature. Excessive dye was removed by washing the cultures four times with distilled water. To quantify matrix mineralization, alizarin red staining was eluted by 100 mM cetylpyridinium chloride for 10 min. The absorbance of the released alizarin red S was measured at 570 nm using a spectrophotometer (Bio-Rad, Hercules, Califonia, USA).

4.8. Statistical analysis

Results were expressed as means ± SD. Data were analyzed by one-way ANOVA, using Bonferroni's modification for post-hoc testing. Comparisons between 2 individual groups were made using 2-tailed unpaired Student's *t*-test. A *p* value less than 0.05 was considered statistically significant.

Results

5.1. Effects of EMD on CTGF expression in unstimulated osteoblastic cells

5.1.1. CTGF gene expression

We examined the effect of EMD on CTGF mRNA expression in serum-free medium with and without 100µg/ml EMD at different time points (6-24h) using RT-PCR (Figure10). Results were expressed relative to control of the same time point. In untreated control cells, CTGF mRNA production was not observed within 6h of culture and was first detected after 12 h. In contrast, EMD-induced CTGF mRNA production was detected within the initial 6h of culture with 100µg/ml EMD. Peak increase in CTGF expression of EMD-induced cells compared to untreated cells was observed at the 12h time point. This time-point was chosen for subsequent experiments. This elevation appeared to decrease from 24h of culture in presence of 100µg/ml EMD.

We then examined a broad range of EMD concentrations for effects on CTGF gene expression. The effect of EMD on CTGF mRNA was dose-dependent over the range of 25-100µg/ml after 12h (Figure 11). EMD at 50µg/ml increased CTGF transcript by 1.6-fold ($p<0.05$), and maximal stimulation was seen at 100µg/ml EMD, where the level of expression was nearly double (1.9 fold) compared with unstimulated controls ($p<0.05$). No significant difference was seen between 25µg/ml EMD-treated group and control.

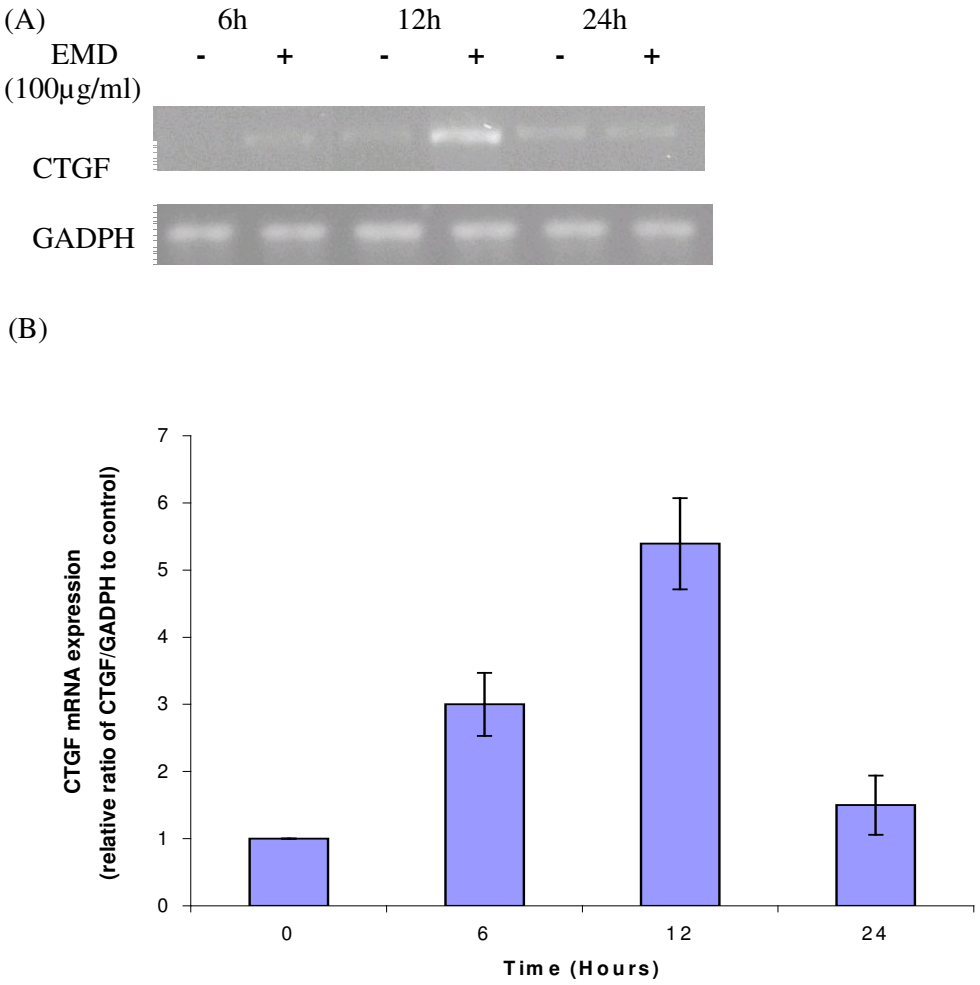


Fig 10. *Time-dependent stimulation of CTGF mRNA by EMD.* (A) One representative gel is shown. GAPDH served to confirm equal loading. (B) The RT-PCR bands were quantified by densitometric analysis and the expression levels of mRNA were normalized to the amount of GAPDH mRNA detected in the same cDNA samples. Results of CTGF mRNA expression by EMD were presented as a relative ratio to CTGF mRNA expression in untreated cells at same time point (relative ratio =1). Data is representative of the response observed in three independent time course studies.

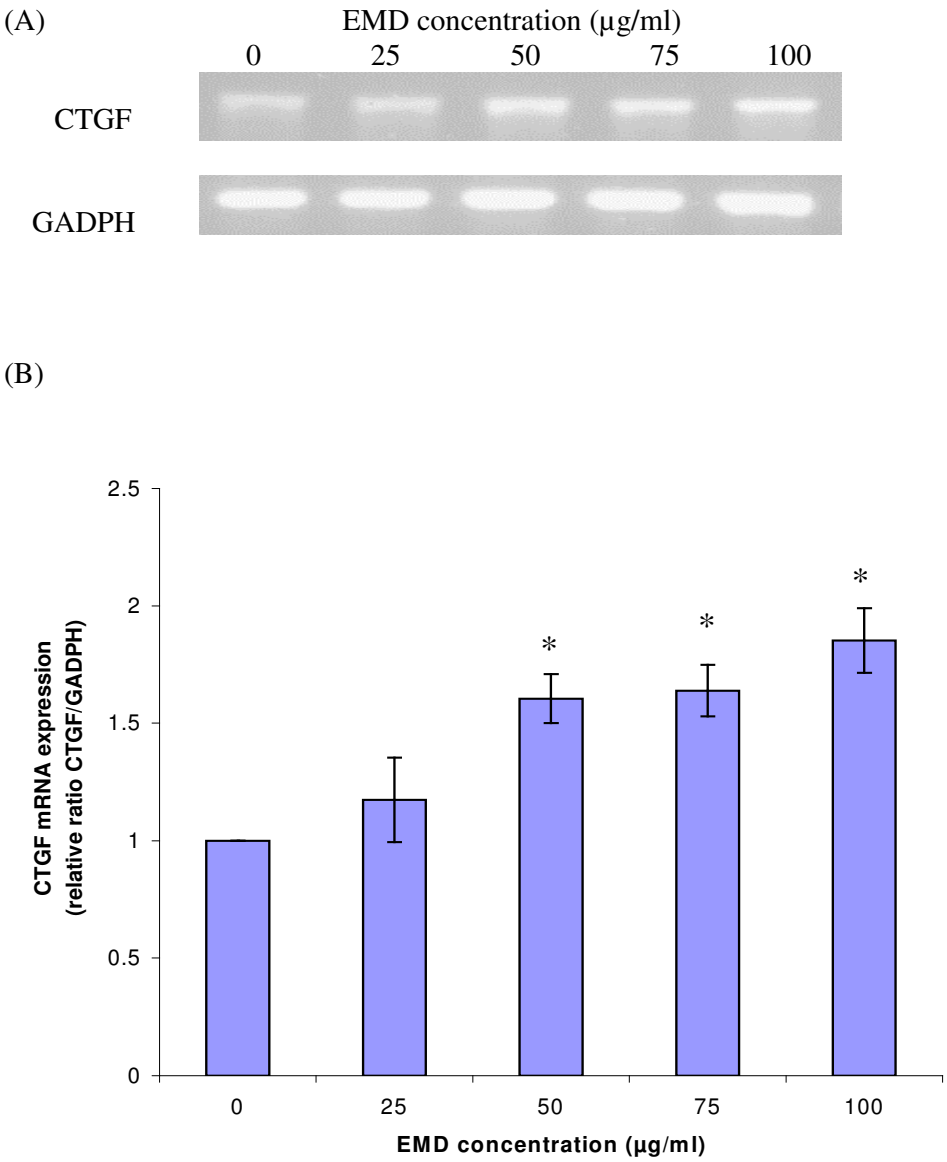


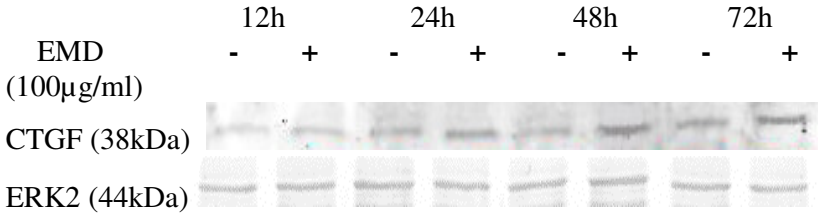
Fig 11. *Dose-dependent stimulation of CTGF mRNA expression by EMD.* Total RNA was extracted after 12h and CTGF mRNA was probed. (A) One representative gel is shown. (B) The RT-PCR bands were quantified by densitometric analysis and the expression levels of mRNA were normalized to the amount of GADPH mRNA detected in the same cDNA samples. Data shown are mean mRNA levels (± SD) relative to untreated control (relative ratio =1.0), of four independent experiments. *$p<0.05$ compared to control.

5.1.2. CTGF protein expression

To confirm that the effects of EMD treatment on CTGF gene expression were reflected at the protein level, Western blot analysis was performed. Quiescent SAOS-2 osteoblastic cells were incubated in 6 cm diameter dishes in serum-free media, with and without 100µg/ml EMD for 12-72 h. Total protein was extracted at indicated times and the 38kDa CTGF protein levels were determined by Western blotting. There was a time-dependent increase in CTGF protein expression treated with 100µg/ml EMD for 12- 48h. This increase appeared to have decreased by 72h of culture (Figure 12). The 48h time point was used for subsequent treatments.

Similar to the effects on CTGF mRNA, a concentration-dependent increase of the CTGF protein levels by EMD was observed in SAOS-2 cells at 48h (Figure 13). EMD, at concentrations of 25µg/ml or greater, increased CTGF protein expression with maximal elevation observed when cells were treated with 100µg/ml of EMD.

(A)



(B)

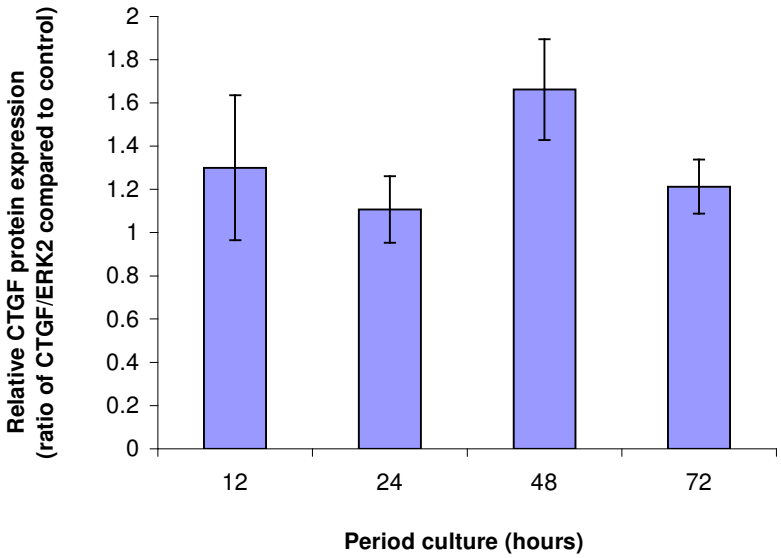
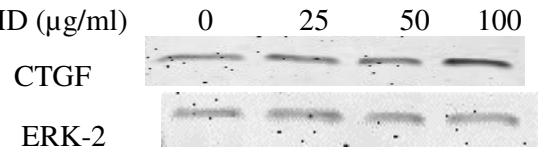


Fig 12. *Time-dependent stimulation of CTGF protein by EMD.* (A) One representative gel is shown. ERK 2 served to confirm equal loading. (B) The bands were quantified by densitometric analysis and the CTGF protein levels were normalized to the amount of ERK 2 protein levels detected in the same lysate samples. Results of CTGF protein expression by EMD were presented as a ratio relative to CTGF protein expression in untreated control cells (relative expression=1) at the same time point. Data is representative of the response observed in three independent time course studies.

(A)



(B)

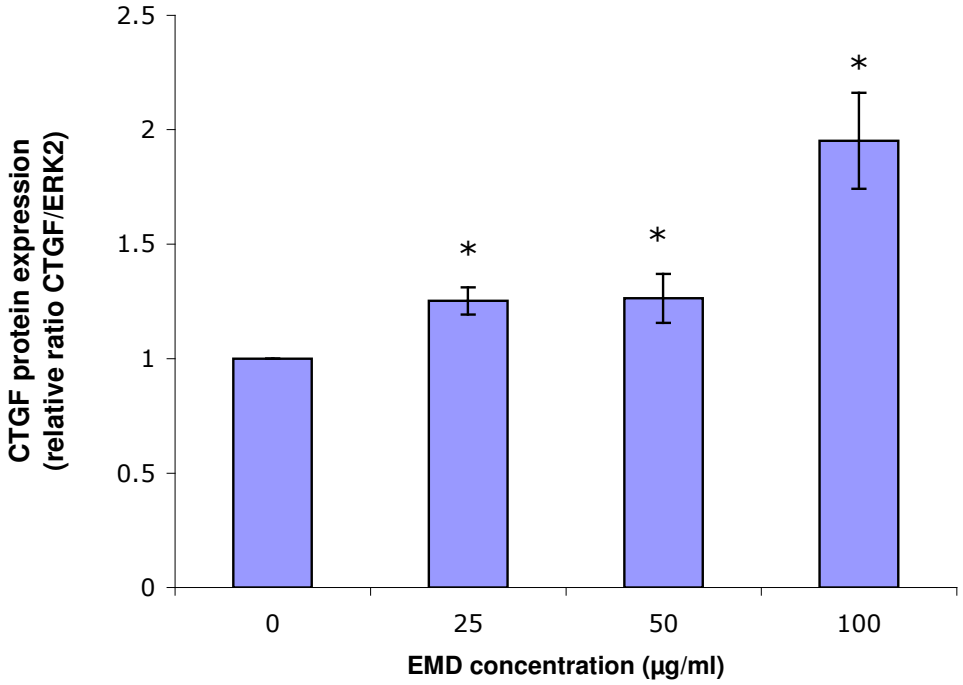


Fig 13. *Concentration-dependent stimulation of CTGF protein expression by EMD.* Total protein was extracted after 48h and CTGF protein was probed. (A) One representative blot is shown. (B) CTGF protein amount was normalized to the amount of ERK2. The data shown is the ratio of treated cultures compared to untreated control (relative expression=1). Three experiments were performed with the same results. *$p<0.05$ compared to control.

5.2. Effects of anti-TGF-ß antibody on EMD-induced CTGF expression

To assess whether EMD-induced CTGF expression is modulated by TGF-ß, we used a well-characterized monoclonal anti-TGF-ß antibody that neutralizes the activity of TGF-ß1, -ß2 and -ß3 specifically without inhibiting the actions of a large number of other cytokines [167]. Approximately 0.01-0.03µg/ml of the antibody neutralizes 50% of the bioactivity due to 0.25ng/ml of TGF-ß1 (R&D systems communication). Based on manufacturer's recommendations, we used 5µg/ml of anti-TGF-ß antibody. We then examined the effects of such antibody on EMD-induced CTGF expression. Untreated cells and cells treated with 5ng/ml TGF-ß1 served as negative and positive controls, respectively.

5.2.1. CTGF gene expression

SAOS-2 osteoblastic cells were incubated in 6-well plates in serum free media, with EMD (100µg/ml) or TGF-ß1 (5ng/ml), with and without anti-TGF-ß antibody (5µg/ml) preincubation. Total RNA was extracted after 12h and probed for CTGF by semi-quantitative RT-PCR analysis. The effect of anti- TGF-ß antibody on EMD-induced CTGF mRNA expression was evaluated by RT-PCR (Figure 14). There was no significant reduction in CTGF mRNA expression when anti-TGF-ß antibody was added alone. In contrast, the antibody significantly inhibited EMD-induced CTGF mRNA production ($p<0.05$; 2.6-fold versus 1.5-fold compared to untreated control). Similarly, the antibody clearly neutralized the effects of TGF-ß on CTGF mRNA expression ($p<0.05$; 7.4-fold versus 1.1-fold).

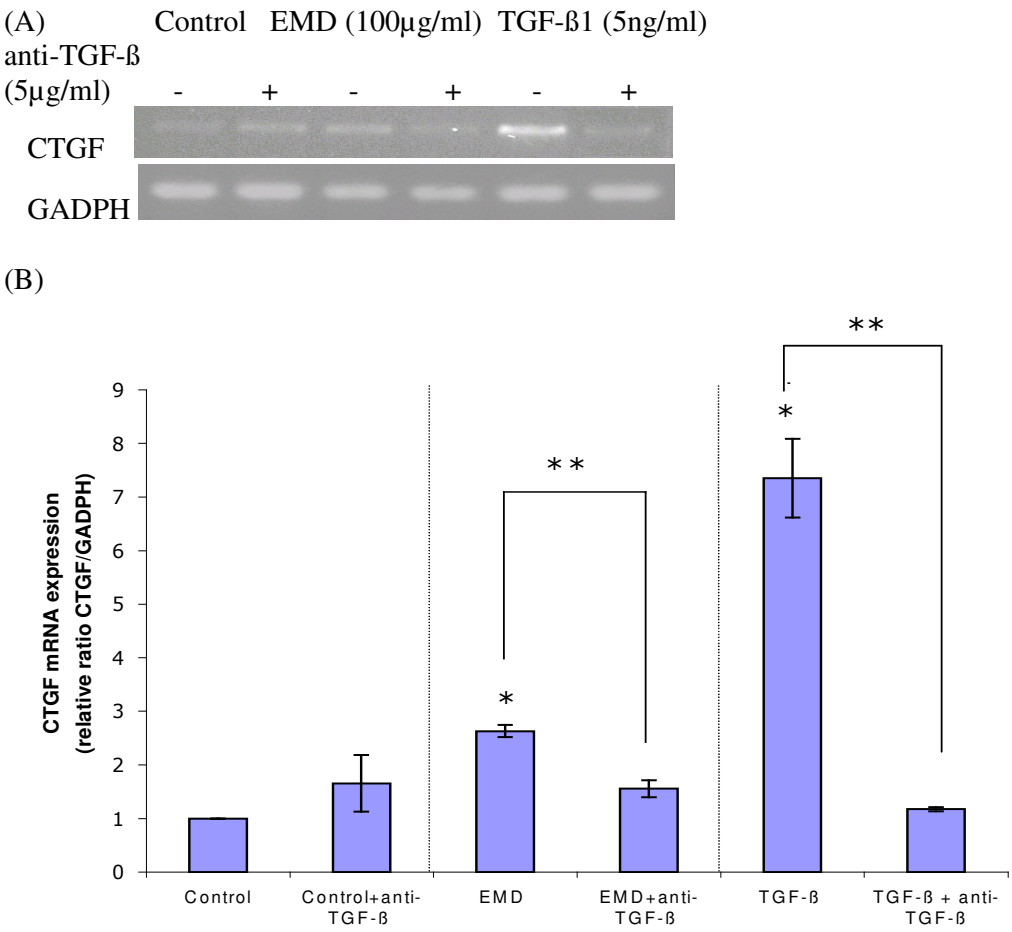
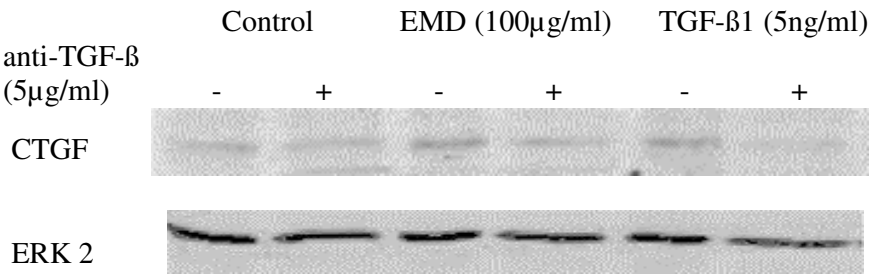


Fig 14. *Inhibition of EMD-stimulated CTGF gene expression by anti-TGF-ß antibody.* Total RNA of SAOS-2 cells was extracted after 12h and probed for CTGF. (A) One representative gel is shown. (B) CTGF mRNA were normalized to the amount of GADPH mRNA detected in the same cDNA samples. Data shown are means of mRNA levels (± SD) relative to untreated control (relative expression=1.0), of three independent experiments. *$p<0.05$ compared to control. **$p<0.05$ compared to same treatment without inhibitor.

5.2.2. CTGF protein expression

Cells were incubated for 48h in serum-free media, with EMD (100µg/ml) or TGF-ß1 (5ng/ml), in the presence or absence of anti-TGF-ß antibody (5µg/ml) preincubation. Similar to RT-PCR findings, the ability of anti-TGF-ß antibody to suppress the induction of CTGF expression was supported by Western blot analysis (Figure 15). In the absence of the neutralizing antibody, CTGF protein expression was enhanced in cultures treated with both EMD (100µg/ml) and TGF-ß1 (5ng/ml). Similar to the RT-PCR findings, the anti-TGF-ß antibody suppressed the induction of EMD-induced CTGF protein levels. The expression decreased in the presence of the antibody in EMD-treated culture ($p<0.05$; 1.64 fold versus 1.19 fold) and an even greater reduction was observed in TGF-ß-treated samples ($p<0.05$; 1.87 fold versus 0.39 fold).

(A)



(B)

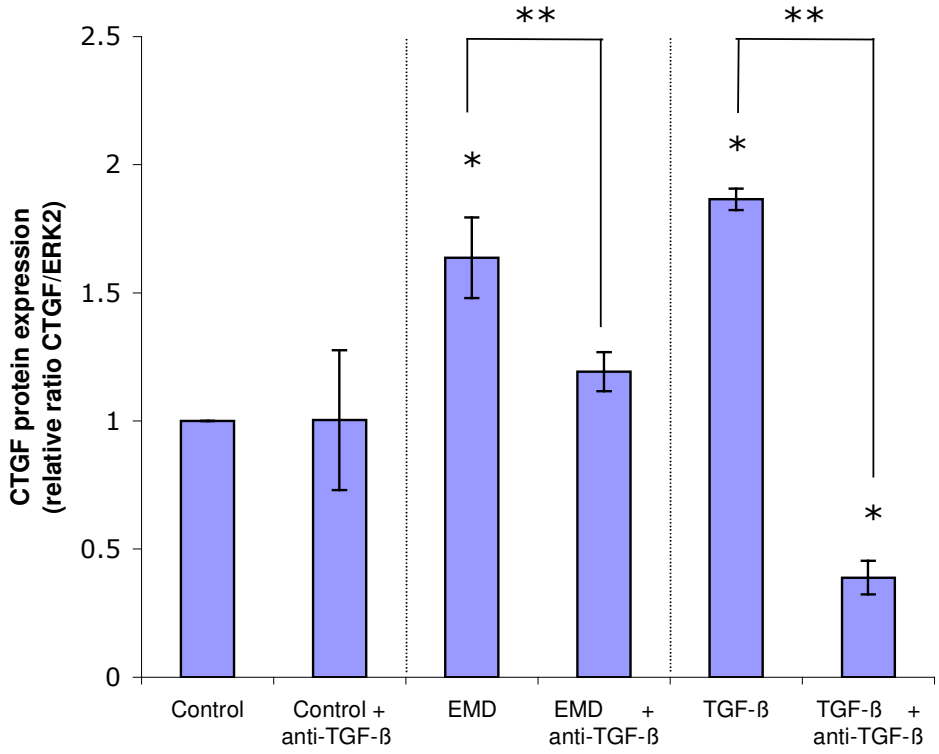


Fig 15. *Inhibition of EMD-stimulated CTGF protein expression by anti-TGF-ß antibody.* (A) One representative blot is shown. (B) CTGF levels were normalized to the amount of ERK2. The data shown is the ratio of treated cultures to untreated control (relative expression=1). Four experiments were performed with the same results obtained. *p< 0.05 compared to control. **p<0.05 with and without inhibitor.

5.3. Effects of CTGF on EMD-induced cell proliferation

Present data revealed an increase of CTGF protein and gene expression by EMD. CTGF has been shown to stimulate the proliferation of osteoblasts [31, 88-90]. Next, we wished to examine the role of EMD-induced CTGF expression in the cell proliferation of osteoblastic cells. Cells were incubated in 2% iFCS-containing media for 48h with various EMD concentrations (0-100µg/ml), with and without anti-CTGF antibody (500ng/ml). The cells were then labeled with BrdU for an additional 2h, and were then subjected to colorimetric immunoassay, as described under the Methods section. We found that BrdU incorporation in proliferating cells was enhanced by EMD treatment compared to control (Figure16). The increase was significant in all EMD concentrations from 25-100µg/ml (p<0.05). However, anti-CTGF antibody (500ng/ml) did not produce any significant effect in BrdU incorporation in each EMD concentration category. Unlike in EMD-treated groups, anti-CTGF antibody had an effect in TGF-ß1 treatment group. (Figure 17).

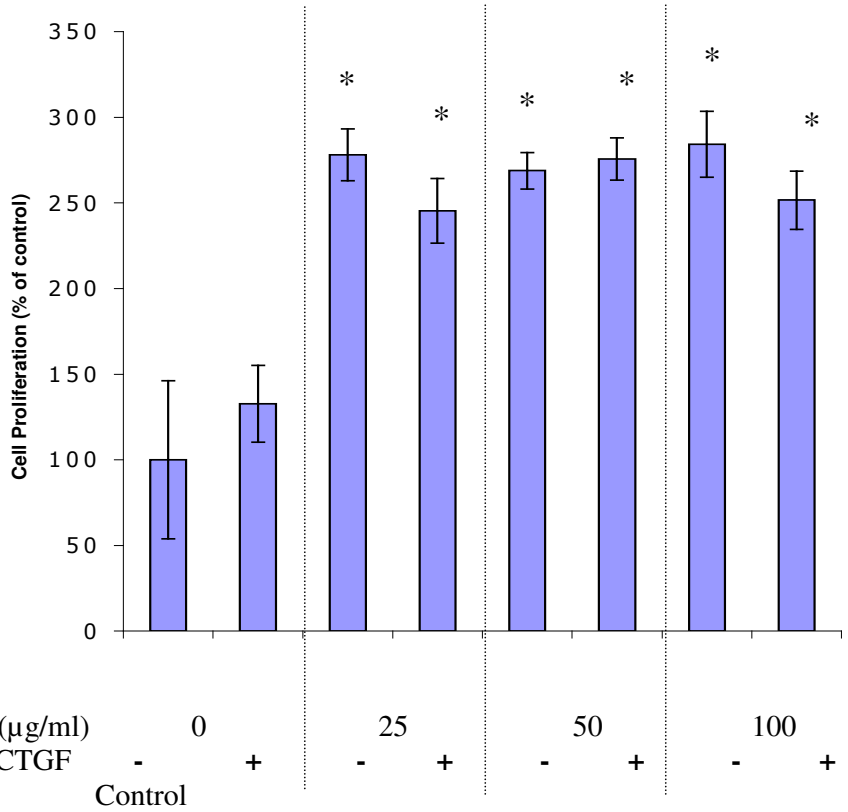


Fig. 16. *Effects of CTGF on EMD-induced DNA synthesis in SAOS-2 osteoblastic cells.* Data shown represents the mean ± SD compared to control of four independent experiments. *p< 0.05 compared to control.

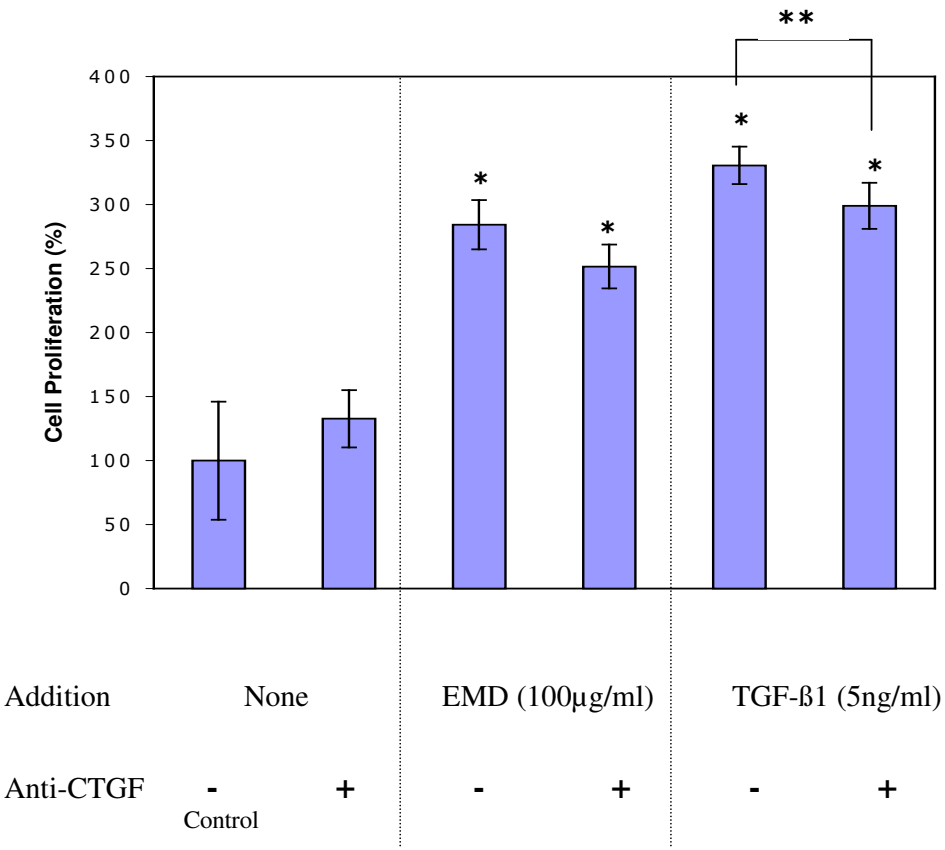


Fig. 17. *Effects of CTGF on EMD- and TGF-ß1- induced DNA synthesis.* Data shown represents the mean ± SD compared to control of four independent experiments. **p*< 0.05 compared to control. ***p*<0.05 with and without inhibitor.

5.4. Cell cycle analysis

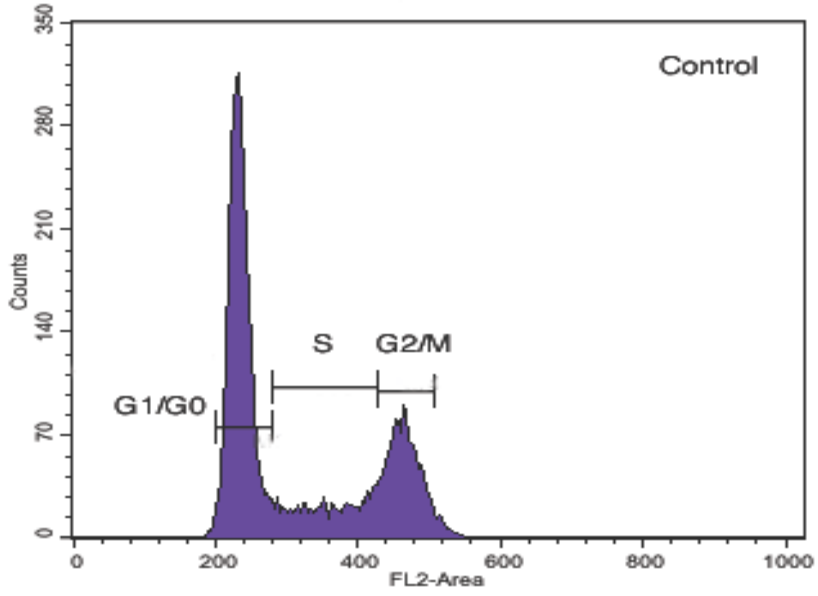
5.4.1. Effect of EMD on cell cycle phases

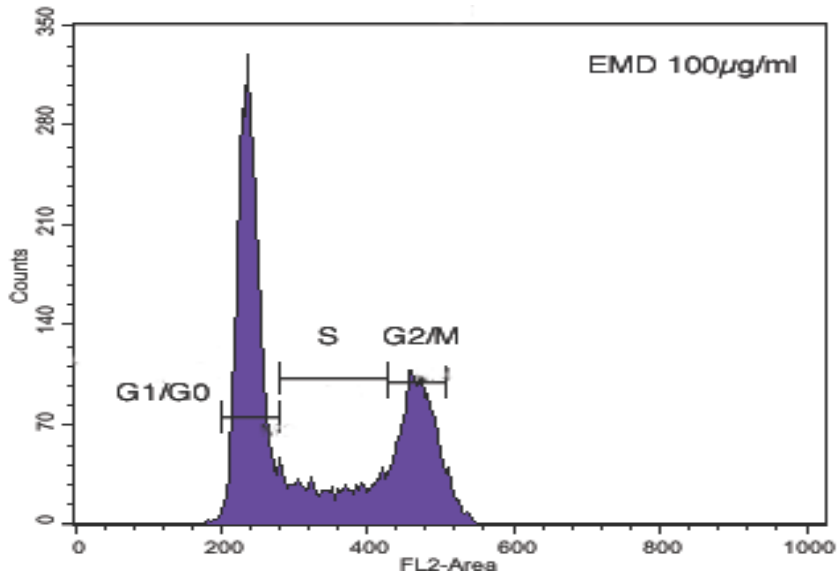
BrdU incorporation experiments demonstrated that EMD significantly induced osteoblastic cell proliferation in dose range of 25-100µg/ml. Therefore we next investigated the effects of EMD on osteoblastic cell cycle progression. Cell cycle analysis in Figures 18 A, B and D demonstrates that EMD had a tendency to dose-dependently increase the percentage of cells in the phase of chromosomal separation and mitosis phase (G2/M phase) dose-dependently. In control cells, an average of 10 % of Saos-2 cells in G2/M phase was observed, whereas in cells treated with 100µg/ml EMD, an average of 17 % of cells was seen. This was accompanied by a reduction of cells going into cell resting phase (G1/G0). The dose dependency trend was observed with no significant difference compared to control.

5.4.2. Effect of CTGF-inhibition on cell cycle in Saos-2 cells

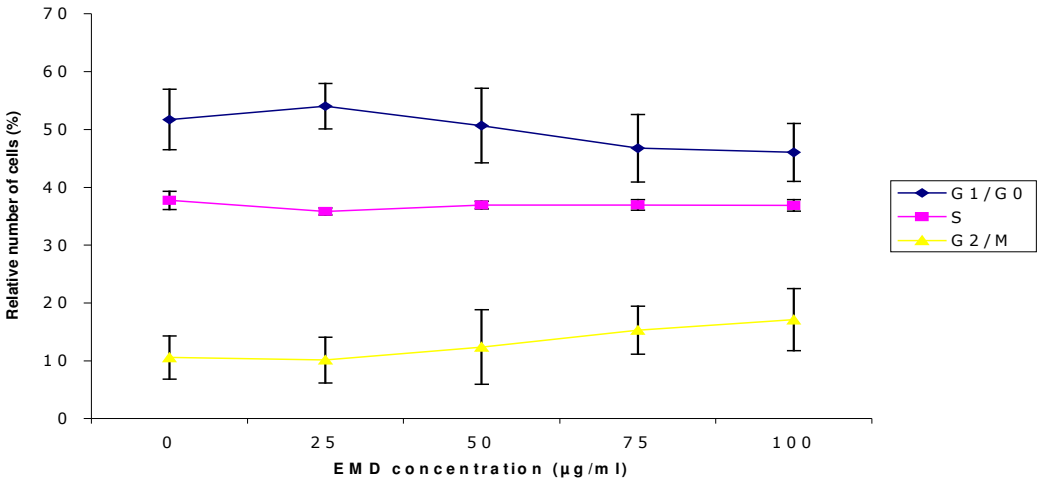
Cells were cultured with EMD (100µg/ml) or TGF-ß1 (5ng/ml), with and without anti-CTGF antibody for 48 hours. Cell cycle analysis in Figures 18 C and D show that, compared to untreated control, cell groups treated with EMD and TGF-ß1 alone (without antibody) showed less cells in resting G1/G0 phase and more cells in both S and G2/M phases. It was found that the addition of CTGF antibody in TGF-ß1 treated cells, and to a lesser extent in EMD-treated cells, resulted in a higher percentage of cells in the resting phase and a lesser percentage of cells in the G2/M phase. No statistical difference was observed.

(A)

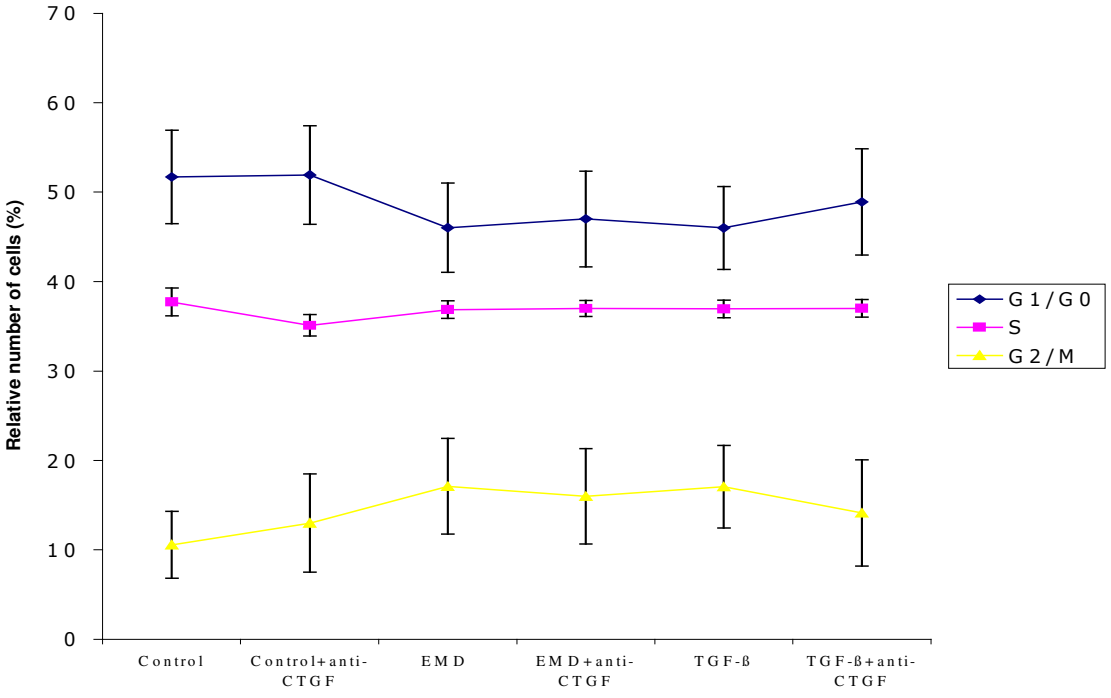

Control
Counts
350
280
210
140
70
0
S
G2/M
G1/G0
0
200
400
600
800
1000
FL2-Area


EMD 100μg/ml
Counts
350
280
210
140
70
0
S
G2/M
G1/G0
0
200
400
600
800
1000
FL2-Area

(B)


70
60
50
40
30
20
10
0
Relative number of cells (%)
0
25
50
75
100
EMD concentration (µg/ml)
G 1/G 0
S
G 2/M

(C)


70
60
50
40
30
20
10
0
Relative number of cells (%)
Control
Control+anti-CTGF
EMD
EMD+anti-CTGF
TGF-ß
TGF-ß+anti-CTGF
G 1/G 0
S
G 2/M

(D)

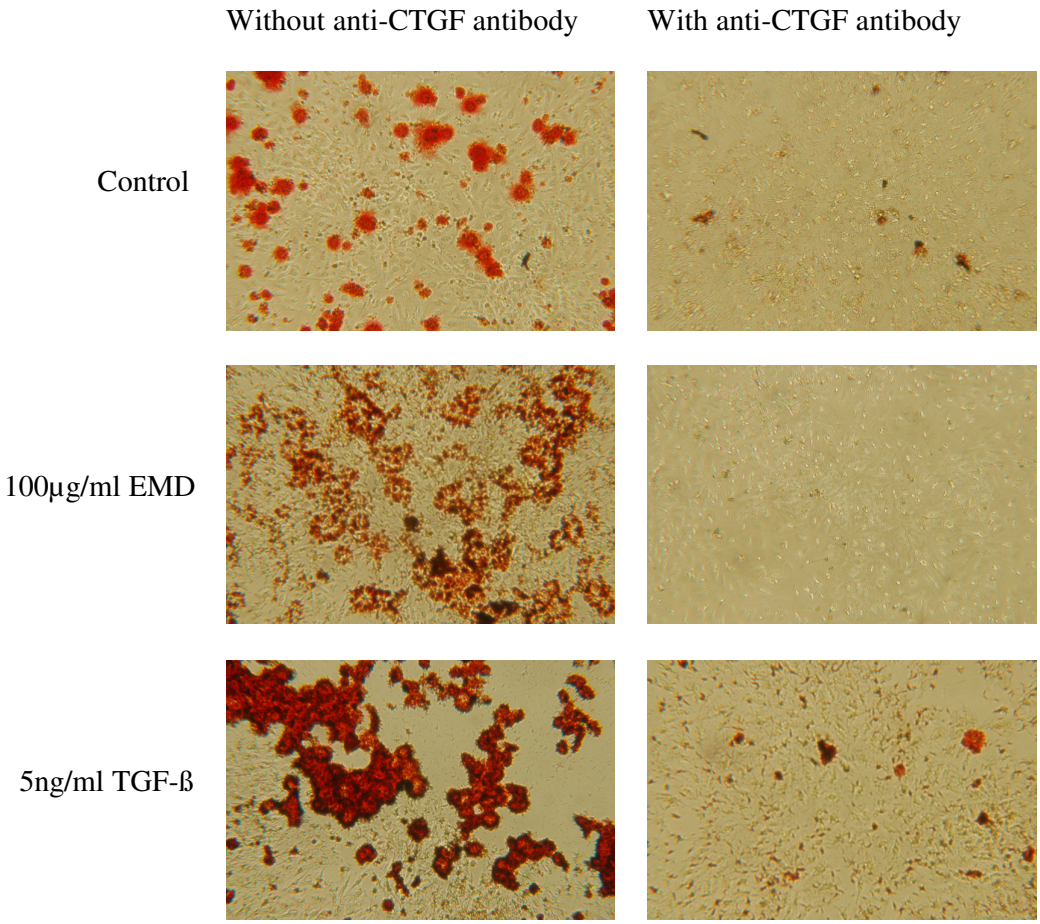
Treatment groups	Cell cycle phase (Mean ± S.D) [%]		
	G1/G0	S	G2/M
No EMD	51.7±5.2	37.7±1.6	10.6±3.7
25µg/ml EMD	54.0±3.9	35.8±0.6	10.2±4.0
50µg/ml EMD	50.7±6.4	36.9±0.7	12.4±6.5
100µg/ml EMD	46.0±5.0	36.9±1.0	17.1±5.4
500ng/ml anti-CTGF	51.9±5.5	35.1±2.1	13.0±5.5
100µg/ml EMD + anti-CTGF	47.0±5.3	37.0±0.9	16.0±5.3
5ng/ml TGF-ß	46.0±4.6	37.0±1.0	16.8±3.7
TGF-ß+ anti-CTGF	48.9±5.9	37.0±1.0	13.6±6.2

Fig. 18 : *The effects of EMD and CTGF on the cell cycle progression.* Shown are the SAOS-2 cells in resting phase (G1/G0 phase), DNA synthesis phase (S) and chromosomal separation and mitosis phase (G2/M), analysed by the flow cytometer. (A) Representative profiles for the DNA content in control versus cells treated with 100µg/ml EMD. (B) Concentration-dependent effect of EMD on the relative number of cells at each cell cycle phase. (C) The effects of anti-CTGF antibody on cell cycles in SAOS-2 osteoblastic cells, where cells were treated with EMD (100µg/ml) or TGF-ß1 (5ng/ml), with and without anti-CTGF antibody (500ng/ml). Each symbol and bar of the graph represents the mean ± S.D. of the data as shown in (D). Data is from six independent experiments.

4.8. Effect of CTGF on mineralization

To investigate the role of CTGF in osteoblastic cell differentiation, the effect on mineralization was investigated by treating the cultures with anti-CTGF antibody. In the presence of the anti-CTGF antibody, there was a significant decrease in mineralization in all cultures ($p<0.05$). Greater inhibition by the antibody was found in both EMD-treated ($p<0.005$; 41% mineralization decrease) and TGF-ß-treated ($p<0.005$; 35% decrease) cultures.

(A)



(B)

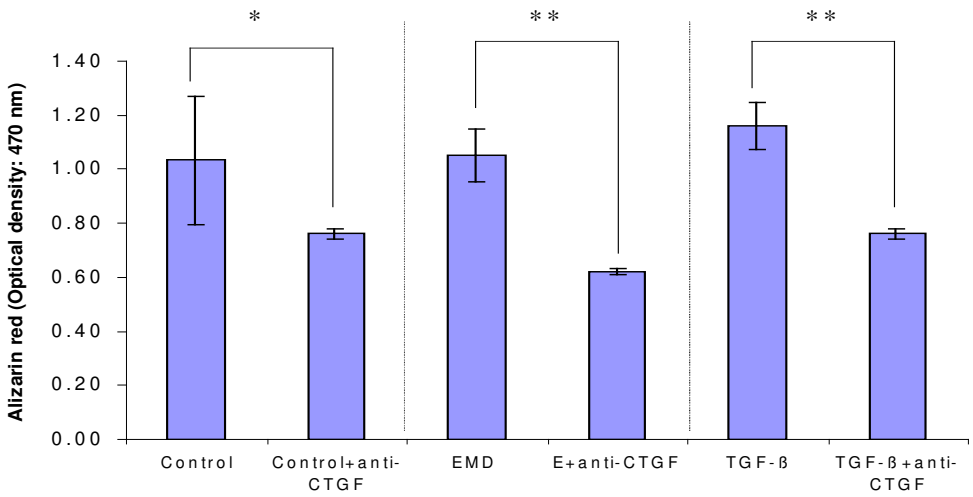


Fig. 19. *Effect of CTGF on mineralization.* Cells were incubated in 2% iFCS-containing differentiation media for 21 days with EMD or TGF-ß1, with and without anti-CTGF antibody. SAOS-2 cell cultures were fixed and stained with 0.5% alizarin red. Subsequently the alizarin red stain was eluted with cetylpyridinium chloride and measured quantitatively.

(A) Photographs show the extent of mineralization (red). Original magnification x100.

(B) Graph shown represents the mean ± SD of three independent experiments. *$p<$ 0.05. **$p<$0.005.

6. Discussion

CTGF has emerged as a potentially important molecule in tissue regeneration and has provided a new target in cell proliferation, differentiation, embryogenesis and wound healing[16, 18]. CTGF is widely recognized as a mediator of TGF-ß. TGF-ß has been shown to be a strong promoter of proliferation and ECM production in osteoblastic cells[71]. It has been suggested that many of the regenerative properties of TGF-ß are actually due to its induction of CTGF production and subsequent stimulation of proliferation and ECM production [23, 26]. Although CTGF is not equivalent to TGF-ß with respect to its biological actions, it functions as a downstream mediator of some of the actions of TGF-ß. TONETTI et al. have reported the up-regulation of TGF-ß and CTGF gene expression in PDL cells treated with EMD [11, 12]. Previous studies have revealed that CTGF stimulates the proliferation and differentiation of osteoblasts, including osteoblastic cells derived from the oral cavity [31, 88-90]. In some studies investigators have found that during odontogenesis, CTGF gene expression has been clearly observed not only in the dental mesenchyme but also in the dental epithelium up to the stage of enamel secretion [91, 92], indicating that CTGF may be involved in the regulation of tooth development. To date, there is no study regarding CTGF expression in osteoblastic cells treated with EMD. For this purpose, we focused on the effects of EMD in the interaction of TGF-ß and CTGF in osteoblastic cells.

EMD has been widely used in periodontal tissue regeneration with promising results [136, 142]. *In vitro* studies show that EMD enhances cellular activities involved in different aspects of tissue regeneration in different cell types. HAMMARSTRÖM et al. have earlier proposed that EMD provides a local environment that is suitable for periodontal regeneration [42]. It promotes osteogenesis and cementogenesis [42]. Exposure to EMD enhances metabolic activity of osteoblastic cells and promotes biosynthesis of ECM molecules [124, 126]. In this context, EMD is thought to induce endogenous production of growth factors and to sustain them at effective doses for a longer period in a local area [9, 10]. In addition, there are studies by KAWASE et al., who

successfully show the presence of TGF-ß1 or TGF-ß1-like factors in EMD [13, 153]. Similarly, it is reported that the derivative contains TGF-ß- and BMP-like growth factors that contribute to the induction of biomineralization during periodontal regeneration [14]. TGF-ß1 plays an important role in the modulation of tissue formation and development of the periodontium [159, 168]. Similarly to EMD [13, 153], TGF-ß1 itself is mitogenic to mesenchymal cells [169] and inhibits epithelial cell proliferation [70]. Hence TGF-ß is considered a potential candidate mediating the effects of EMD. Although the interactions between EMD and TGF-ß on osteoblastic cell growth and bone formation have been well described, the interactions between EMD and CTGF, a downstream mediator of TGF-ß, are still unknown. The primary goal of the study is to examine the short-term effects of EMD in human osteoblastic cells on CTGF expression. Using this approach, we examine whether EMD has stimulatory effects on CTGF expression in the osteoblastic cell model. The present data suggests that, EMD up-regulates CTGF protein and gene expression in human osteoblastic cells via TGF-ß-dependent pathway. Also EMD-induced matrix mineralisation, but not proliferation, is affected by CTGF.

6.1. Discussion of methods and materials used

6.1.1. Choice of osteoblastic cell line

We have chosen Saos-2 cell line as a model of human oral osteoblastic cells, because of its good and well-documented characterization [170-172]. Saos-2 cells originated from a primary human osteogenic osteosarcoma of an 11-year-old Caucasian woman. It is reported that the cells exhibit the entire differentiation sequence of osteoblastic cells, namely proliferation, matrix formation and mineralization [173]. It is well recognized that Saos-2 cells have many features of a moderately to well-differentiated osteoblastic cell type and this includes high tissue non-specific ALP activity and an ability to mineralize their matrix when cultured in mineralizing medium[170, 172, 174]. Hence this makes these cells a valuable model for studying events associated with proliferation and differentiation stages in osteogenesis. Saos-2 cells also grow faster than primary cultures of normal osteoblastic cells and large amounts of cells can be obtained in a short time. It exhibits

good stability of most phenotypic properties over approximately 100 passages, with differences being only quantitative in nature and not exceeding a factor of 2 [175]. Furthermore, working with primary osteoblast preparations always brings with it the possibility of non-osteoblastic cell contamination, which can contribute to or even dominate the effects under investigations. This is because primary osteoblastic cell preparations can be difficult to isolate and cannot be expected to be homogeneous. This is especially the case in the periodontium, which consists of a wide array of cell types such as gingival epithelial cells, PDL fibroblasts, cementoblasts and odontoblasts, making specific cell isolation difficult. Moreover, Saos-2 cells can be used in medium containing minimal FCS (2% serum content) or even in serum-free medium [176]. This minimizes confounding variables due to animal-derived proteins, such as growth factors, found in the serum. This is an important factor in our study, as we would like to examine the effects of EMD on growth factor expression and proliferation. Addition of serum would otherwise introduce exogenous soluble mediators, such as cytokines and growth factors, into our experimental medium that would then affect the validity of our study. Moreover, Saos-2 cells have been shown to significantly express growth factors, especially TGF-ß1, IGF and BMPs, and respective growth factor receptors [177, 178]. The cells are reported to have the ability to richly express mRNA for TGF-ß1 [177] and the type V TGF-ß receptor, specific for TGF-ß1, is found in such cells [178]. More importantly, it has been verified that CTGF receptor are found on such cells [31]. In contrast, to date, there is no study yet showing the presence of CTGF receptor in human primary osteoblastic cells. Besides, Saos-2 cells have been shown to express CTGF in little amounts during resting stage but can highly express CTGF when appropriately stimulated, making the cells a useful model of assessing factors affecting CTGF changes[90, 174, 179]. Hence, with the above considerations in mind, we decided to choose Saos-2 cell line to examine whether EMD has stimulatory effects on CTGF expression.

6.1.2. Serum content for cell growth prior to kinetic analysis

In earlier studies by GESTRELIUS et al. (1997), a growth medium with 10% FCS content was used [4]. It is well known that the cellular morphology and cell growth are affected *in vitro* by essential factors found in serum components [180]. However, commercially available FCS contains a non-specific content of serum components and mitogenic factors. In order to avoid unpredictable reactions due to serum components, the serum concentration to assess cell proliferation should be kept as low as possible. In our experiments on proliferation and mineralization, a low FCS concentration at 2% was selected, with similar effects observed in higher serum concentrations. This low concentration has worked particularly well in investigations associated with bone regeneration and osteoblastic cells [181, 182].

6.1.3 RT-PCR

Semi-quantitative RT-PCR serves as an initial important series of experiments where we have first verified the presence of CTGF mRNA production in EMD-treated and untreated cells under our experimental conditions.

Choice of CTGF primer for DNA sequencing

One of the single most important factors in successful DNA sequencing prior to RT-PCR is proper primer design. Criteria [183-185] we considered for selecting appropriate primer included: (1) selecting a primer size from 18 to 30 bases in length, (2) having a melting temperature (Tm) in the range of 52^{o}C to 65^{o}C, (3) having an absence of significant hairpin formation, (4) having guanine-cytosine (GC) content of 40% to 60%. The CTGF primer was designed with computer assistance using Primer 3 (Whitehead Institute for Biomedical Research). Our chosen oligonucleotide sequence was further verified in nucleic acid sequence database (Pubmed's Blast program) to ensure that the sequence was indeed complementary to CTGF gene sequence. We chose the CTGF primer sequences (forward and reverse) as shown in Table 4 because they

exhibit the following advantages: (1) they have primer sizes of 20 and 19 bases, respectively, (2) they have a melting temperature of 63^{o}C, (3) they do not possess self-complementarities, that is, there is little chance of them forming secondary primer structures (eg hairpin formation) and primer dimers, (4) they have a GC content of 55-60%, (5) the primer sequences have also been previously used to examine CTGF expression and induction by TGF-ß [186].

6.1.4 Western Blotting

The investigation on mRNA raises the next question i.e., whether the effects observed on the transcriptional level would also have an influence on translational protein level. Protein synthesis involves a complex network of intracellular mechanisms. After translation, many proteins still have to undergo numerous post-translational modifications, such as phosphorylation and glycosylation, before becoming functional. Western blot, together with RT-PCR, is a crucial method to clearly demonstrate whether major qualitative changes in CTGF mRNA expression induced by EMD are expressed on the translational protein level as well.

6.1.5 BrdU cell proliferation assay

We have chosen colorimetric BrdU ELISA assay to study cell proliferation because, compared to the autoradiographic detection of [^{3}H]-thymidine incorporated into DNA, it has the advantages of speed and convenience of analysis, and it obviates the need to use radioisotopes. The BrdU method is similar in specificity and sensitivity to the autoradiographic detection of [^{3}H]-thymidine labeled nuclei[187, 188]. The immunocytochemical detection of BrdU incorporated into DNA is a powerful tool to study the kinetics of the cell. BrdU is incorporated into the newly synthesised DNA of the S-phase cells and can thus provide an estimate for the fraction of cells in S-phase and the rate of proliferation. BrdU is often used with FACS analysis using PI staining in order to provide a clearer image of the cell cycle [189].

6.1.6 Cell cycle analysis using FACS

One of the uses of cell cycle analysis using flow cytometry is to assess cell proliferation. There is an intimate relationship between cell cycle and cell growth. Proliferation is usually accompanied by a lesser proportion of cells in the resting G1/G0 phase with more cells involved in DNA synthesis (S phase), DNA replication (G2 phase) and mitosis (M phase). Cell cycle analysis by the effects of EMD has so far been poorly studied. A single study was carried out by KAWASE et al. [153], who examined the effects of EMD on the cell cycle in various human oral epithelial and fibroblastic cell lines (oral epithelial SCC25 cell line, gingival fibroblastic Gin-1 cell line, PDL primary cell line and epithemoid carcinoma-derived A431 cell line). They found that EMD substantially stimulated the proliferation of both PDL and Gin-1 cells dose-dependently, but EMD failed to modulate A431 epithelial carcinoma cell proliferation. EMD was found to inhibit epithelial cell proliferation in SCC25 cells by reducing the relative proportion of cells at proliferative S and G2 phases and increasing the percentage of cells in G1 resting phase. Through methods other than cell cycle analysis, other studies also show that EMD has a cytostatic action on epithelial cells [4, 9]. Similarly, TGF-ß has been shown to inhibit proliferation and induce the apoptosis of epithelial cells [70].

To date, there is no study which examines the effect of EMD on cell cycle in osteoblastic cells. The advantage of FACS over direct cell counting is that the latter uses a hemocytometer to manually determine cell number and growth, which can lead to observer fatigue if it takes a long time to count the proliferating cells [189]. Hence we decided to examine the cell cycle and cell growth of osteoblastic cells treated with EMD using FACS analysis, and use this method to assess whether the addition of anti-CTGF antibody had an effect on the osteoblastic cell cycle. This is achieved by using PI as the cell cycle stain. The dye passes through intercalates into cellular DNA. The intensity of the PI signal is directly proportional to DNA content. Hence FACS analysis using PI is commonly used to determine the proportion of cells in various stages of the cell cycle.

6.1.7 FACS versus BrdU proliferative assay

Three principal periods of osteoblast developmental sequence are cell proliferation, matrix development and maturation, and mineralization [34]. It has been well established in many studies that the principal role of CTGF is that of osteoblast development and differentiation [18, 31]. However, effects of CTGF on osteoblast proliferation are still questionable, where its role in proliferation may be secondary [18, 190]. Hence, in our study we chose to measure the effects of CTGF in EMD-induced proliferative phase.

The effects on EMD on cell cycle analysis have so far been poorly studied. As there is no other study which uses FACS to study the effects of EMD on osteoblastic cells, we decided to use this method to assess the cell cycle in Saos-2 osteoblastic cells, with particular focus on finding out more about cell proliferation. For comparison of sensitivity and reliability of methodology, we also carried out the more widely used enzyme-linked immunoassay (ELISA) to assess proliferation. We decided to use BrdU proliferation assay because this ELISA assay has been one of the most widely used methods to find out the effects of EMD in cell proliferation [9, 13, 153]. The assay has been shown to work particularly well in investigations associated with bone regeneration and osteoblastic cells [125, 191]. Hence to examine the potential of the flow cytometry assay as an alternative assay for the assessing cell proliferation in EMD-treated osteoblastic cells, we compared and contrasted it with BrdU proliferative immunoassay. Both methods assess cell growth conveniently. Compared to BrdU, which only assesses DNA synthesis, FACS analysis provides a more rounded overall picture of the cell cycle. It shows the amount of cells in each critical stage of the cell cycle, that is, in the resting phase, DNA synthesis phase, chromosomal separation phase and in mitotic phase. However a major disadvantage of FACS is its lower sensitivity compared to ELISA [192-194]. NECKERS et al. compared BrdU method *versus* PI in assessing DNA synthesis. They found that the percentage of cells in S phase calculated by the BrdU method was higher than that calculated by the PI method. It is postulated that FACS using PI is less sensitive and is not capable of distinguishing an increase in total DNA of less

than 5% [195]. These studies support our study where although we found that FACS using PI successfully show result trends similar to those of BrdU ELISA method, the former produces comparably higher variability in values, with higher standard deviations compared to what we have found using BrdU ELISA. Another disadvantage we found in FACS procedure is that it is more time consuming compared to BrdU ELISA. Besides time needed for fixing the cells and incorporating PI, the analysis using the flow cytometer also takes longer than spectrometer analysis used in ELISA. This is because for reliable sorting, even on a high-speed sorter, the flow rate cannot exceed a few thousand cells/second, resulting in low rate of throughput [196, 197]. Besides it being more time consuming, more cell yield is needed for FACS analysis compared to ELISA. This is due to a possible loss of yield. According to a review regarding various methods to determine cell proliferation by BARNES at al., on average, in about one quarter of cases, the DNA histograms produced cannot be interpreted if there are many incomplete or poorly preserved nuclei [189]. These pathologic nuclei could also be due to cell processing prior to FACS and therefore, the integrity of cell morphology can be lost [189]. Other disadvantages of FACS compared to ELISA include high cost of FACS machine and ease of use. Hence despite its shortcomings, we have found that FACS provides a clear image of the cell cycle and is useful to get an overview of the trend of effects observed. The broad spectrum of information it provides nicely complements the more in-depth sensitive BrdU ELISA for assessment of proliferation.

6.1.8 Alizarin red staining

Besides proliferation, we would also like to look into the effects of CTGF in the late stage of osteoblastic development, such as bone formation. In this study we examined the extent of mineralized nodule formation by Alizarin red staining and the alizarin red stain was subsequently eluted with cetylpyridinium chloride for quantitative analysis. Alizarin red S staining has been used for decades to evaluate calcium-rich deposits by cells in culture. It is particularly versatile in that the dye can be extracted from the stained monolayer and assayed for matrix mineralization.

6.2. Discussion of results

6.2.1. Effects of EMD on CTGF expression

6.2.1.1. Time-course of CTGF mRNA and protein expressions induced by EMD

The present study indicates a time-dependent stimulation of CTGF expression by EMD in osteoblats, with the first detection of CTGF mRNA production within the initial 6 hours of culture with 100µg/ml EMD and with peak increase in CTGF expression observed at the 12th hour time point. This elevation appeared to decrease from 24 hours of culture. These findings parallel to those reported on the gene expression profiling of periodontal ligament (PDL) cells stimulated with EMD. The study revealed that addition of EMD to PDL cells *in vitro* elicited a rapid substantial up-regulation (>2.0 relative increase) in CTGF RNA synthesis, which was found to occur within the first 2 hours of culture with EMD [12]. However no change in CTGF gene expression in EMD-stimulated cells compared to that of control cells was observed after 24 hours. In addition, TGF-ß was reported to be up-regulated within 2 hours but no change in TGF-ß gene expression in EMD-stimulated cells compared to that of control cells was observed at 24 hours [12]. On the protein level, the expression of CTGF protein was highest at 48 hours. This may be secondary to the prolonged activation of ERK pathway and of Smad phosphorylation, mechanisms involving TGF-ß signaling pathway [198]. Previous studies have demonstrated that EMD rapidly produced phosphorylation of MAPK family, such as ERK, and activation of Smad1/2 pathway and such effects were sustained for over 24 hours [13, 199].

6.2.1.2. Concentration-dependent effects of EMD on CTGF expression

We found that EMD enhanced CTGF gene expression, especially at higher EMD concentrations (from 50µg/ml). On the protein level, we also observed similar findings regarding the up-regulation of CTGF protein in high EMD concentration of 100µg/ml. This indicates that the stimulatory changes found in CTGF transcriptional stage are translated onto the protein level. Such changes may be attributed to the presence of TFG-ß1. BRETT et al. and PARKER et al.

used cDNA arrays comprising a wide variety of genes coding for growth factors, cytokines and their receptors, to elucidate changes in gene expression in human PDL cells exposed to EMD [11, 12]. The PDL cells, derived from freshly extracted teeth, were treated with 100µg/ml of EMD for 24 hours [12] and for 4 days [11]. They found that both TGF-ß and CTGF genes were up-regulated. PARKERS et al. found that CTGF gene was up regulated with a 2.4 relative increase after 4 days of culture with EMD [11]. Their findings using cDNA array method verify to what we have found in our study, where we used RT-PCR to analyze CTGF gene expression after treatment with EMD.

6.2.1.3. EMD affects CTGF expression via TGF-ß pathway

TGF-ß is recognized as a potent inducer of CTGF. Since EMD has been reported to contain TGF-ß-like growth factors and stimulate the levels of TGF-ß1 endogenously, we postulated that EMD affects CTGF expression via TGF-ß pathway. To investigate the mechanism behind EMD actions, we used an anti-TGF-ß antibody. TGF-ß1 served as a positive control in our experiments, and we have found that TGF-ß1 alone strongly induced both CTGF mRNA and protein expressions. More importantly, TGF-ß1 was also used to test the validity and reliability of the anti-TGF-ß antibody.

In the present study, the addition of the anti-TGF-ß antibody significantly inhibited both EMD-induced and TGF-ß1-induced CTGF gene expression to near basal levels. This demonstrates the ability of anti-TGF-ß antibody to significantly modulate the EMD/CTGF interaction and specifically to override the potent induction of CTGF by TGF-ß pathway. This suggests that activation of TGF-ß signaling pathway forms the basis of CTGF expression and induction by EMD. According to a review by JENSSENS et al. [71], molecular signaling by the TGF-ß members involves the Smad-dependent and MAPK-dependent pathways. For the latter, the MAPKs which are particularly correlated strongly with TGF-ß signaling are ERK, JNK and p38 MAPK. *In vitro* studies have found that increasing EMD concentrations lead to increasing amounts of TGF-ß1 [10, 13, 14, 124, 126]. SCHWARTZ et al. studied the effect of EMD on TGF-ß1

production in MG63 human osteoblast-like osteosarcoma cells by treating confluent cultures with 0, 25, 50 and100 µg/ml EMD for 24 hours and total TGF-ß1 released into the medium was measured using ELISA. It was found that EMD caused a dose-dependent increase in TGF-ß1 with cultures treated with more than 50µg/ml EMD [126]. YONEDA et al. found that EMD strongly enhanced mRNA expression of collagen I in a dose-dependent manner in KUSA/a1 mouse osteoblastic cells after 4 days of culture in EMD concentrations up to 50µg/ml. EMD was also found to enhance TGF-ß1 mRNA expression in similar EMD concentrations (≥ 50µg/ml) [191]. It has been hypothesized that EMD not only forms an insoluble matrix that induces endogenous production of growth factors by regenerative cells, it also contains TGF-ß-like molecules [13, 14, 153]. KAWASE et al. found the presence of TGF-ß-like molecules in EMD preparations. EMD was subjected to enzyme-immunoassay for TGF-ß1 and it was found that EMD preparations contained TGF-ß1-like immunoreactivity that bonded to TGF-ß receptor II. They also found that increasing EMD concentrations (0-200µg/ml) exerted a dose-dependent effect on the levels of TGF-ß1 or TGF-ß1-like substances [153]. Several studies have shown that EMD activates of both MAPK and Smad signaling pathways [13, 153, 200, 201]. KAWASE et al. found that EMD (25-100µg/ml) rapidly produced dose-dependent phosphorylation of MAPK family: ERK, p38 MAPK and JNK in both oral epithelial and fibroblastic human cells. EMD also rapidly stimulated translocation of Smad 2 into the nucleus of both cell types. He suggested that TGF-ß might act as a principal growth regulating agent in EMD despite being present in only low levels [153]. Although our study did not investigate the Smad and MAPK-dependent pathways directly, we have demonstrated that anti-TGF-ß antibody could strongly block the up-regulation of EMD-induced CTGF expression. This cellular outcome may be a consequence of the activation of the Smad and MAPK-dependent TGF-ß signaling pathways. This interesting possibility can be further investigated in future studies.

6.2.2. Role of CTGF in EMD-induced osteogenesis

It has been proposed that CTGF is involved in osteoblast proliferation and differentiation[18]. NISHIDA et al. have reported that in Saos-2 human oral osteosarcoma cell line and the MC3T3-E1 mouse osteoblastic cell line, the effect of CTGF on the proliferation and differentiation of osteoblastic cells is stimulatory [31]. Since TGF-ß1 has been shown to stimulate osteoblast proliferation and TGF-ß1 up-regulates CTGF expression, we would like to find out whether CTGF may mediate the stimulatory effects of TGF-ß1 on osteoblasts. We observed that addition of neutralizing anti-CTGF antibody inhibited TGF-ß-induced proliferation in osteoblastic cells. This could be because cellular responses by TGF-ß1, such as cell proliferation, are induced directly and indirectly via CTGF-dependent pathways [97, 202] (Figure 19). Hence it seems likely that CTGF-dependent pathway has a role in the proliferative actions of TGF-ß1.

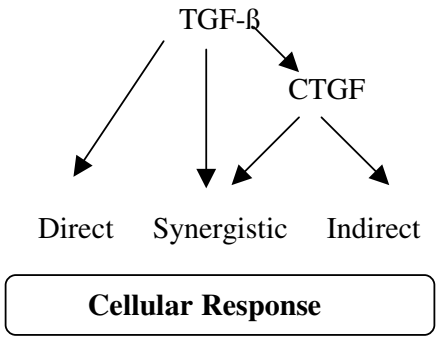


Fig 20 A model illustrating the potential pathway for TGF-ß and CTGF signaling. Cellular responses may be induced directly by TGF-ß, including CTGF gene expression, or indirectly via CTGF action.

In our findings from both FACS and BrdU assays, Saos-2 osteoblastic cells responded to various concentrations of EMD by increased proliferation. In support of our findings, numerous previous studies have also described comparable proliferative responses after EMD stimulation [9, 10, 125, 126, 191, 203-206]. In bone cells, EMD stimulates proliferation and promotes differentiation and mineralization in relatively mature osteoblasts [124-126, 207, 208]. In relation to our study in using the same cell line, SCHWARZ et al. studied the effect of EMD on Saos-2 cells on titanium implants

where attachment, proliferation and viability of such osteoblasts were investigated. It was concluded that EMD enhanced cell proliferation and viability of human Saos-2 cells on titanium implants in a concentration-dependent manner [209].

We next investigated the possible involvement of CTGF in the proliferation in EMD-stimulated osteoblastic cells. We observed that the proliferative effect of EMD was not attenuated by anti-CTGF antibody. In our BrdU findings, EMD induced a significant increase in proliferation (more than twice as much) at a wide range of concentrations (25-100µg/ml), with and without the inhibition of CTGF. Therefore, these findings indicate that the proliferative action of EMD on osteoblastic cells is not substantially affected by direct pathways which are CTGF-dependent. Although the beneficial effects of EMD on osteoblastic cell proliferation and differentiation are well recognized, the mechanisms of action are still being debated. It has been demonstrated that EMD functions as an insoluble matrix to promote cells to produce growth factors [3, 148, 149]. There is also another hypothesis according to which bioactive molecules released from EMD are also responsible for the tissue regenerative activity of EMD. The bioactive molecules could be growth factors absorbed to EMD during its preparation or amelogenin peptides [4, 13, 154, 155, 158, 159]. A major component of EMD contains proteins belonging to the amelogenin family [4, 158], which stimulates PDL and bone cell proliferation and differentiation [210, 211]. In addition to the larger major form of amelogenin, multiple amelogenin gene product fragments also exist as a result of alternative splicing [154, 155]. VEIS et al. have reported that these small amelogenin peptides have been shown to actively participate in cell signalling to stimulate matrix formation and mineralization and are believed to have osteogenic activities [154, 155, 157]. Moreover, there are also other growth factors that may be responsible for the stimulating effects of EMD. It has been widely hypothesized that EMD not only induces TGF-ß but also induces or contains other growth factors such as BMP, IGF and PDGF [9, 11, 12, 14, 124, 159]. Hence, as EMD contains a large array of factors, such as various growth factors and amelogenin peptides associated with the stimulatory effects of EMD, it is still unknown whether solely TGF-ß and CTGF alone are

present in sufficient amounts to elicit a significant direct effect in proliferation. Moreover as proliferation is a highly regulated process, which involves the interaction of a wide array of signaling molecules with different regulatory profiles, cell proliferation is regulated by a wide variety of other factors, besides TGF-ß and CTGF. Hence the interactions of a wide variety of mitogenic factors induced by EMD contributes to the overall proliferative actions of EMD.

On the other hand, we found that in the presence of anti-CTGF antibody, all cell cultures exhibited a significant reduction of mineralisation. It is postulated that in bone formation, CTGF may play a more principal role in osteoblast development and differentiation than in cell proliferation [18, 31]. Studies have suggested that CTGF promotes collagen I synthesis, osteopontin, osteocalcin and alkaline phosphtase, which are markers of bone differentiation [18, 30, 31]. In our study, the observed greater inhibitory effect by anti-CTGF antibody on matrix mineralization compared to untreated cells, indicates that CTGF plays a role in EMD-induced and TGF-ß-induced osteoblastic cell differentiation. To our knowledge, this is the first study demonstrating CTGF as a major mediator of EMD-induced matrix mineralisation.

7 Conclusion

In conclusion, our study suggests that EMD stimulates CTGF expression in osteoblastic cells and such interaction is modulated via the TGF-ß pathway. In addition, CTGF plays an important role in EMD-induced osteoblastic matrix mineralization but not proliferation. These results provide a new insight on the EMD-CTGF interaction, two biomodifiers that have therapeutic relevance to tissue engineering and regeneration.

TGF-ß and CTGF are considered as crucial growth factors involved in tissue regeneration. Although TGF-ß1 and CTGF are potential candidates mediating the effects of EMD, their presence and participation in EMD-induced tissue regeneration have not yet been clearly defined. Our findings show that EMD induces CTGF expression through TGF-ß pathway. Greater understanding of the exact TGF-ß-dependent signaling pathways (such as Smads and MAPK pathways) is needed to provide more detailed molecular and mechanistic information on the effects of EMD on CTGF. Further study using primary osteoblastic cells and other types of cell lines, such as fibroblastic and epithelial cell types, will be useful to provide a better picture on whether such effects are cell-specific. This will provide a newer insight into the interactions of various growth factors and EMD and the underlying mechanisms involved that will ultimately have therapeutic relevance to periodontal regeneration, where EMD can be used as a modulator of regenerative growth factors in oral tissue engineering.

8 Summary

Enamel matrix derivative (EMD) stimulates the production of transforming growth factor-beta (TGF-ß), which is suggested to play a role in mediating the effects of EMD in periodontal tissue regeneration. Connective tissue growth factor (CTGF) is a mediator of TGF-ß and promotes cell development. The interaction between EMD and CTGF is still unknown. This study explores the effects of EMD on CTGF expression in human osteoblastic cells and whether the interaction is modulated by TGF-ß signaling pathway. Also, the roles of CTGF in cell proliferation, cell cycle progression and mineralized nodule formation of EMD-induced osteoblastic cultures are examined.

Human osteoblastic cells (Saos-2) were treated with 25-100 µg/ml EMD with or without the addition of TGF-ß inhibitor. CTGF mRNA expression was detected by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and CTGF protein levels were assayed by Western Blot analysis. In addition, cell cycle progression and DNA synthesis were determined by flow cytometry (FACS) and BrdU incorporation following EMD treatment, with or without CTGF antibody. Mineralisation was examined by alizarin red staining and quantified by elution with cetylpyridinium chloride.

Western blot and RT-PCR analysis demonstrated a dose-dependent increase of CTGF expression by EMD. EMD-induced CTGF expression was significantly reduced in the presence of TGF-ß inhibitor. Both cell cycle and BrdU analysis revealed an increase of cell proliferation following EMD treatment, due to an increase of the percentage of cells in the G2/M phase of the cell cycle. No significant effect was found when anti-CTGF antibody added. On the other hand, in the presence of the anti-CTGF antibody, mineralization was significantly inhibited in EMD-treated cultures.

The present study demonstrates that EMD stimulates CTGF expression and the interaction is modulated via TGF-ß in osteoblastic cells. Also, CTGF affects EMD-induced osteoblastic

mineralization but not cell proliferation. These results provide a novel insight on the EMD-CTGF interaction, two biomodifiers that have therapeutic relevance to tissue engineering and regeneration.

9. Zusammenfassung

Schmelzmatrix Proteinderivate (EMD) simulieren die Produktion des Wachstumsfaktors Transforming Growth Factor-beta (TGF-ß), das die EMD-induzierten Effekte in der parodontalen Regeneration maßgeblich kontrolliert. Connective Tissue Growth Factor (CTGF) ist ein Mediator des TGF-ß, das die Zellproliferation und extrazelluläre Matrixproduktion fördert. Der Einfluss von EMD auf die CTGF Expression in Osteoblasten ist jedoch nicht bekannt. Aus diesem Grund ist das Ziel dieser Studie den Effekt von EMD auf die Expression von CTGF sowie den Einfluss von TGF-ß in humanen Osteoblasten zu untersuchen. Außerdem wird die Bedeutung von CTGF für die EMD-induzierte Zellproliferation, Zell Zyklus Progression und Mineralization analysiert.

Humane Osteoblasten (Saos-2) wurden mit 25-100µg/ml EMD mit und ohne Zusatz von 0.1µg/ml TGF-ß Inhibitor in serumfreien Kulturmedium behandelt. Die CTGF mRNA Expression wurde mittels RT-PCR analysiert. Die CTGF Proteinexpression wurde mittels Western Blot Analyse untersucht und densitometrisch quantifiziert. Um den Effekt von CTGF auf die EMD-induzierte Zellproliferation bzw. Progression des Zellzyklus zu ermitteln, wurden die Zellen mit 25-100µg/ml EMD mit und ohne Zusatz von 0.5µg/ml CTGF Inhibitor behandelt und mittels BrdU ELISA sowie mittels FACS Analyse untersucht. Die Effekt auf die EMD-induzierte Mineralization wurde mittels Alizarin Rot Färbung untersucht und densitometrisch quantifiziert.

Die RT-PCR und Western blot Analysen zeigen eine konzentrationsabhängige Zunahme der CTGF mRNA und Proteinexpression durch EMD. Die Zugabe des TGF-ß Inhibitors reduzierte signifikant die EMD-induzierte CTGF Expression, was auf eine Modulation der EMD-induzierten CTGF Expression über die TGF-ß Signalkaskade hindeutet. Die BrdU Analyse zeigte eine 2,5-fache Stimulation der DNA Synthese durch EMD, allerdings hatte die Zugabe des CTGF Inhibitors keinen Effekt. Dies konnte mittels FACS Analyse bestätigt werden. Die Alizarin Rot Analyse zeigte, die Zugabe des CTGF Antikörper reduzierte signifikant die EMD-induzierte Mineralization.

Zusammenfassend stimuliert EMD die CTGF Expression und diese Interaktion scheint durch TGF-ß moduliert zu werden. CTGF hat eine Effekt in die EMD-induzierte Mineralization aber die EMD-induzierte Stimulation der Zellproliferation wird allerdings nicht durch CTGF reguliert. Diese Ergebnisse liefern einen neuen Einblick in die Interaktion zwischen EMD und

CTGF, zwei Biomodifikatoren, die eine entscheidende Bedeutung für das Tissue Engineering und die parodontale Regeneration haben.

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Erklärung

„Ich, Nora Heng, erkläre, dass ich die vorgelegte Dissertationsschrift zum Thema: *The Effects of Enamel Matrix Derivatives on the Regulation of Connective Tissue Growth Factor (CTGF) in Human Osteoblastic Cells*, selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."
Berlin, den 27/02/07

Nora Heng