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The role of platelet-derived growth factor in the development of mouse molars

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ABSTRACT Platelet-derived growth factor (PDGF) is a potent mitogen that functions in cytodifferentiation and wound healing. PDGF receptor-α (PDGFR-α) is required for the normal development of the dental ectomesenchyme (Stephenson *et al., Proc. Natl. Acad. Sci. USA 88:* 6-10, 1991). To investigate the regulatory potential of PDGF on tooth development, the expression of PDGF-AA was determined by reverse transcription-polymerase chain reaction (RT-PCR), the PDGF ligands and receptors were localized by immunohistochemistry. The growth-promoting effects of exogenous PDGF-AA on molar explants were determined by assaying for tritiated thymidine incorporation, the presence of type I collagen and amelogenin messenger RNA transcripts, and total DNA, RNA and protein accumulation. Based upon the temporal and spatial localization, PDGFs and their receptors are present in the enamel epithelia and pulpal mesenchyme of developing mouse molars. Exogenous PDGF-AA administration increased the total protein accumulation of mouse molar explants but produces no discernible effect on amelogenin and type I collagen expression.

KEY WORDS: *PDGF, PDGF receptor, immunolocalization, RT-PCR, pulp cell culture, tooth development*

Introduction

Platelet-derived growth factor (PDGF) is an essential epigenetic signal during embryonic development as well as the principal regulator of tissue regeneration in adult life (Alberts *et al.*, 1989; Stephenson *et al.*, 1991). In humans the PDGF ligand is encoded by two distinct genes. PDGF-AA (125 amino acids) is expressed from a gene located on chromosome 7 (Stenman *et al.*, 1992a) while the PDGF-BB (160 amino acids) is from a gene located on chromosome 22 (Dalla Favera *et al.*, 1982; Stenman *et al.*, 1992b). These two polypeptides share 56% amino acid identity. The two PDGF chains associate to form a dimeric molecule which is processed proteolytically on both its amino- and carboxyl-termini. The active PDGF ligands range in molecular weight from 28 to 35 kDa and are secreted either as AA or BB homodimers or as the AB heterodimer (Ross *et al.*, 1986). PDGF-BB is more potent than PDGF-AA as a mitogenic and chemotactic agent (Ferns *et al.*, 1990; see review Heldin, 1993). Upon binding to its specific cell surface receptor, PDGF stimulates a signal transduction pathway leading to DNA synthesis and protein production. The PDGFR (Mr= 160 to 185 kDa) is a member of a larger family of receptor protein-tyrosine kinases which includes colony-stimulating factor receptor (CSF-1-R) and fibroblast growth factor receptor (FGFR). Structurally, these receptors contain an extracellular amino-terminal ligand binding domain, a short hydrophobic single pass transmembrane domain, and an intracellular carboxyl-terminal tyrosine kinase catalytic domain interrupted by a polypeptide insertion. In humans the two PDGFR genes (PDGFR-α and PDGFR-ß) are located on chromosomes 4 (Stephenson *et al.*, 1991) and 5 (Matsui *et al.*, 1989) respectively. The PDGF receptor displays a complexity similar to that of its ligand. The PDGF receptors form active dimers upon ligand binding creating up to three isoforms: αα, ßß and αß. The PDGFR-α binds all three PDGF isoforms, PDGFR-αß binds PDGF-AB as well as PDGF-BB and the PDGFR-ß binds only PDGF-BB (Heldin *et al.*, 1988; Nister *et al.*, 1988). The various functional regions of the PDGFRs show different levels of conservation. The structural complexity of the PDGF ligand/receptor system is likely to correspond to a parallel functional complexity such that the three dimeric receptor isoforms exhibit some uniqueness in their association with other regulatory or catalytic elements of the signal transduction cascade.

Abbreviations used in this paper: PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; CSF-1-R, colony stimulating factor 1 receptor; FGF-4, fibroblast growth factor-4; FGFR, fibroblast growth factor receptor; E, embryonic day; PN, post natal day; +C, positive control; –C, negative control; RT-PCR, reverse transcription-polymerase chain reaction; Dix-1, Distalles-1 gene; TGF β, transforming growth factor beta; BMP-2, bone morphogenic protein-2; EGF, epidermal growth factor; LEF1, lymphoid enhancer factor 1.

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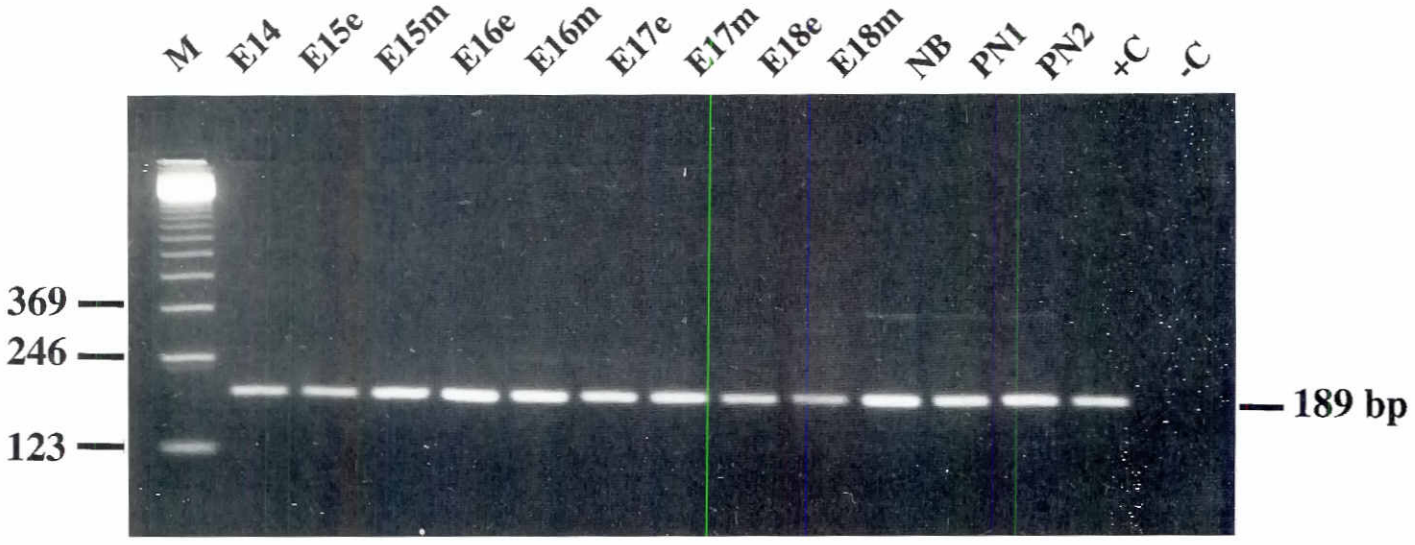

Fig. 1. RT-PCR amplification of PDGFA mRNA (189 bp) isolated from enamel organ epithelia (e) or dental mesenchyme (m) of E15 through PN2 mouse mandibular first molars. *The entire tooth germ was used from E14 embryo. The first lane (marked M) is the 123 bp DNA ladder (GIBCO BRL, Gaithersburg, MD, USA). The size of the marker bands (in base pairs) is provided on the left. The positive control marked as +C was mRNA from fetal liver tissue. The last lane (marked -C) is a negative control in which the PCR reaction contained no mRNA. NB: new born; PN1: post natal day 1; PN2: postnatal day 2.*

In mice a recessive embryonic lethal mutant known as Patch *(Ph)* has been characterized as a deletion in platelet-derived growth factor receptor alpha (PDGFR-α) gene (Stephenson *et al.*, 1991; Morrison-Graham *et al.*, 1992). A few *Ph/Ph* embryos survive past embryonic day 10 (E10) and exhibit significant connective tissue malformations. These connective tissue defects are the result of decreased mesenchymal cell proliferation as well as decreased and abnormal extracellular matrix production. In one-third of the *Ph/Ph* mutants the enamel organ did not form and the typical condensation of dental papillae was not observed. This suggests that PDGF/PDGFR is required for the normal development of the dental ectomesenchyme. The normal physiological functions of PDGF reveal its potential as a growth-promoting factor. It has been used to enhance tissue regeneration in a number of clinical studies. The application of PDGF-BB for treatment of pressure-induced ulceration and chronic, non-healing, cutaneous ulcers has shown significant improvement in epithelialization and connective tissue repair without toxicity (Knighton *et al.*, 1990; Robson *et al.*, 1992; Haynes *et al.*, 1994). PDGF enhances the local formation of cartilage and bone after subcutaneous implantation of demineralized bone matrix (Howes *et al.*, 1988) and, when combined with insulin-like growth factor-I in post-implant insertion and periodontal surgery, favored new bone formation and periodontium regeneration.

The complex process of odontogenesis depends upon precise epithelial-mesenchymal interactions that are controlled by multiple transcription factors, growth factors, receptor molecules, structural components of cells and extracellular matrix components (see reviews by Ruch, 1995 and Thesleff *et al.*, 1995). In the present study we investigate the role of PDGF in tooth development. The temporal-spatial expression of PDGFs and their receptors in odontogenic tissue was tested using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. PDGF was used as a media supplement in maintaining developing mouse mandibular first molar explants and molar pulp cells. The cultures were assayed for tritiated thymidine incorporation, total DNA, RNA and protein content, and for the expression of type I collagen and the enamel specific protein, amelogenin.

Results

PDGF and PDGF receptors are both expressed in developing mouse molars. PDGF-AA mRNA transcripts could be detected in the enamel organ and the dental papilla mesenchyme during the bud and cap stages of odontogenesis and subsequently in the dental pulp and the ameloblast layer of bell stage molars (Fig. 1). These results demonstrate the existence of PDGF-AA RNA messages in the developing murine molars from E14 through PN2. The PDGF receptor was not detected in the dental epithelium by immunostaining late E14 molars but was present in the dental mesenchyme of E17 (bell stage) molars (Fig. 2A-C). At late bell stage PDGF receptors localized to both the ameloblast layer and pulpal mesenchyme. The signal for the receptors was most intense in vascular structures, alveolar bone matrix, preodontoblasts and differentiating odontoblasts (Fig. 2A-C). The pattern of PDGF staining was similar to PDGFR in the cap and bell stage molars (Fig. 2A,E; D,F). The ligand was most concentrated during the cap stage in the oral epithelium and less abundant in the alveolar bone matrix and enamel organ epithelia (Fig. 2E). At a later (bell) stage its distribution was detected in both epithelial and mesenchymal structures (Fig. 2F). RT-PCR analysis detected the presence of PDGF-AA transcripts prior to the localization of the protein by immunohistochemistry. The sensitivity of RT-PCR as well as the physiological appearance of mRNA prior to protein synthesis could account for this difference.

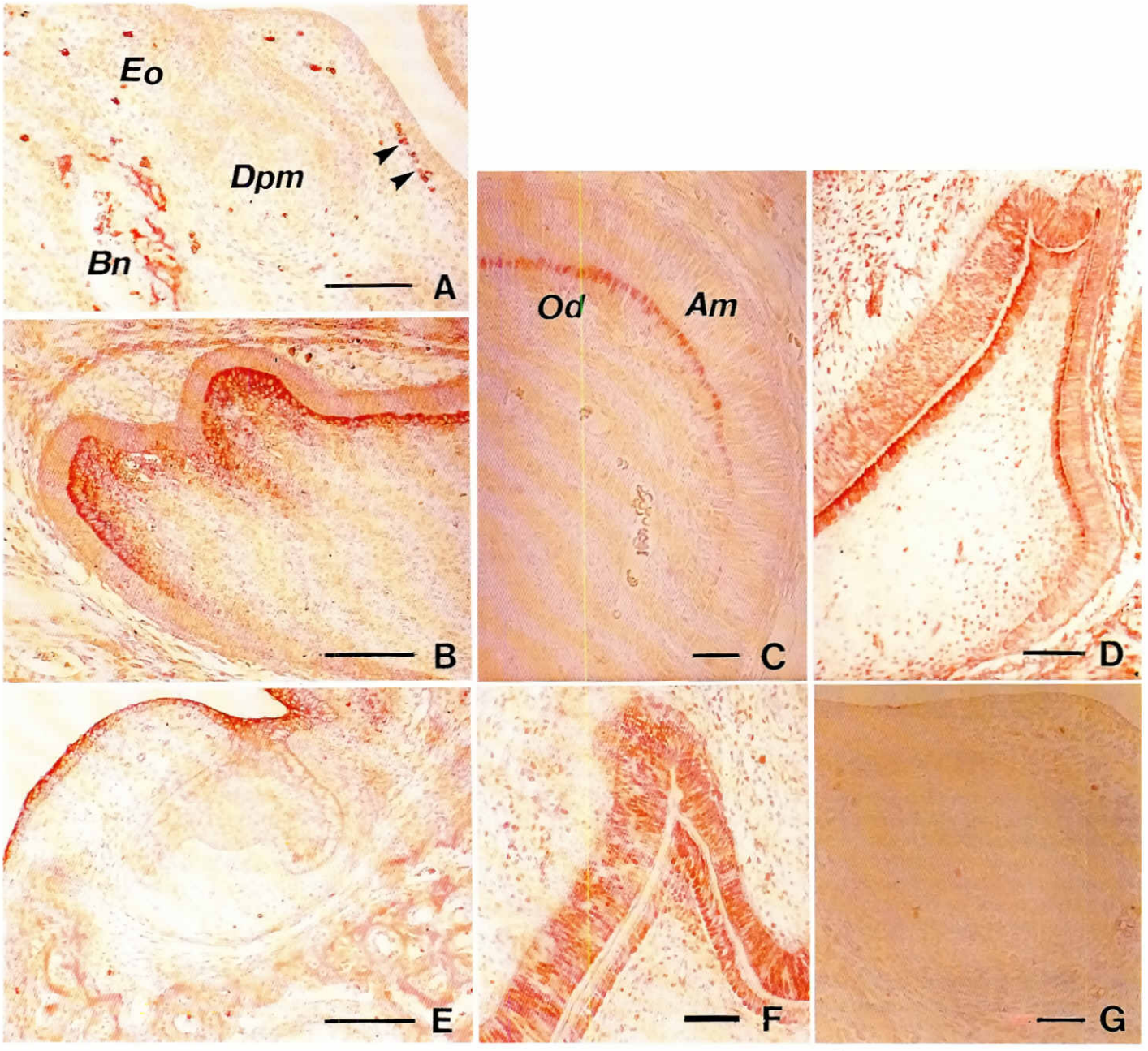

Fig. 2. Immunolocalization of PDGFRs and PDGFs in developing mouse mandibular first molars. *The early cap stage (E14) molar showed positive signal of PDGFRs in vascular spaces and alveolar bone matrix* **(A)** *and at late E18 PDGFRs were mainly associated with the differentiating odontoblasts* **(B)**. *The first detection of PDGFRs was localized on odontoblasts and the underlying dental pulp from E17 first molar* **(C)**. *The PDGFRs were detectable in most of the dental tissues at crown completion stage, PN2* **(D)**. *The PDGF ligands were present mainly in the E14 oral epithelium, alveolar bone matrix, enamel epithelium* **(E)** *and later in the differentiating ameloblasts and odontoblasts of PN1 molar* **(F)**. *Negative control sample of a E14 molar presents no positive staining* **(G)**. *Am: ameloblasts; Eo: enamel organ; Od: odontoblasts; Dpm: dental papilla mesenchyme; arrowheads: vascular space, Bn: alveolar bone. Bar, 100 μm.*

PDGF-AA was used to supplement the media of cultured tooth organs. Although supplementation ranged from 0.25 ng/ml to 25 ng/ml, only at a concentration of 2.5 ng/ml were differences discerned between treatment groups and controls. Exogenous addition of 2.5 ng/ml of PDGF-AA increased cell proliferation and total protein accumulation in developing mouse molars without an increase in total DNA or RNA content (Fig. 3). No differences were observed in mRNA transcript levels for amelogenin, type I collagen, or PDGF-AA in explants treated with PDGF-AA (Fig. 4).

The effects of PDGF-AA and PDGF-BB supplementation were measured on rat pulp cells grown in culture. PDGF-AA

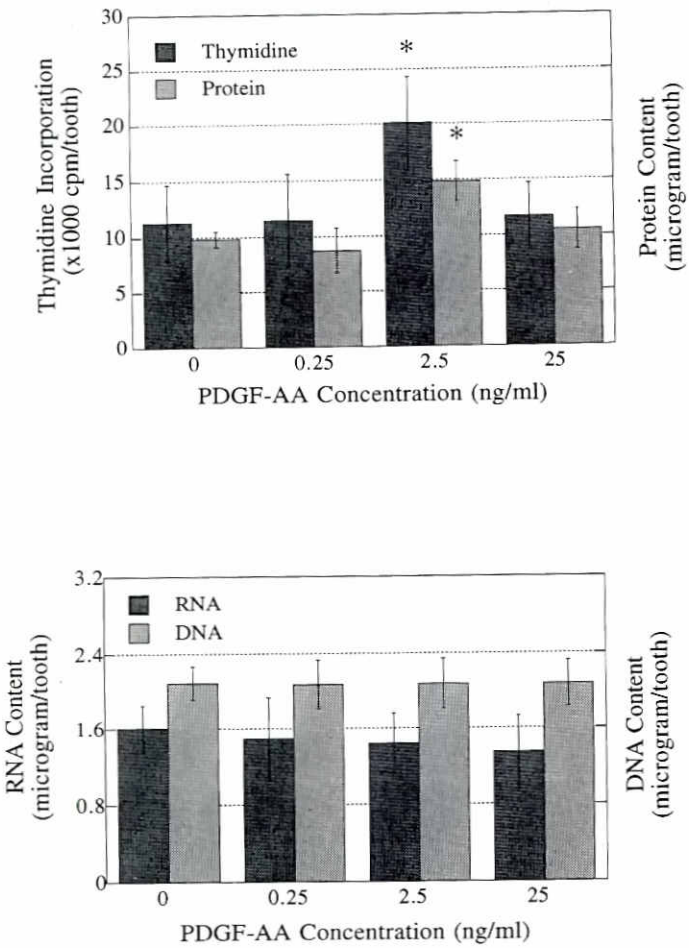

Fig. 3. Changes in thymidine incorporation, protein, DNA and RNA content in mouse mandibular first and second molar explants following exogenous PDGF-AA administration for 7 days *(7 dc-days in culture). Asterisk denotes statistic significance at p<0.05. PDGF-AA at concentration of 2.5 ng/ml increased cell proliferation and total protein accumulation in the developing mouse molars with no significant increase of the total DNA and RNA content.*

and PDGF-BB at a concentration of 25 ng/ml increased the rate of cell proliferation with no net change in total protein content (Fig. 5).

Discussion

The induction of odontogenesis is associated with the expression of multiple transcription factors such as the homeobox genes Msx1, Msx2, Dlx-1, Dlx-2, and LEF1. Among the multitude of target genes are growth factors such as FGF-4, TGFß, BMP-2, BMP-4, EGF, PDGF, as well as the receptors for these factors (for a recent review see Thesleff *et al.*, 1995). These molecules are involved in intercellular communication and amplify the input by inducing transcription factors that influence the expression of a large number of genes downstream. These signaling molecules are also involved in a multitude of developmental processes so that a genetic defect at this level produces far ranging abnormalities. *Patch* mutants in mice are caused by a defect in the PDGF alpha receptor. While the condition is lethal the mice survive long enough to display tooth agenesis (Morrison-Graham *et al.*, 1992). In this study we investigate the role of PDGF signaling in cell proliferation and the secretion of the major components of the dentin and enamel extracellular matrices.

Finding PDGF ligand and receptor in separate but adjacent structures is a general feature of mouse embryogenesis (Orr-Urtreger and Lonai 1992; Orr-Urtreger *et al.*, 1992; Schatteman *et al.*, 1992) and is suggestive of epigenetic signaling by a paracrine mechanism. The action of PDGF during odontogenesis does not strictly follow this pattern. The mRNA for PDGF-AA was detected throughout odontogenesis in both the enamel epithelium and dental mesenchyme and corresponded to the distribution pattern of the PDGF protein. Early in odontogenesis (through the early bell stage) the PDGF receptor was only expressed by differentiating odontoblasts. These observations indicate that PDGF signaling during tooth formation does not adhere strictly to a reciprocal pattern. The expression of PDGF ligand in both epithelial and mesenchymal tissues while the receptor is confined to the mesenchyme suggests that PDGFR restricts the effects of a more pervasive PDGF signal to the mesenchyme. The unique association of PDGFRs with differentiating odontoblasts suggests a possible role for PDGF in the regulation of odontoblast differentiation.

The effects of exogenous PDGF-AA on culture molars were measured for ligand concentrations ranging from 0.25 to 25 ng/ml. Changes were only observed at 2.5 ng/ml. At this concentration PDGF-AA increased both tritiated thymidine incorporation and total protein accumulation. This finding is similar to the report by Nakashima that PDGF stimulated extracellular matrix proteoglycan production as well as [^{125}I]-deoxyuridine incorporation in cultured pulp cells and suggesting that PDGF regulates the activities of differentiating pulp cells (Nakashima, 1992). The expression of the major enamel and dentin matrix proteins, amelogenin and collagen type I, did not vary with the PDGF supplementation, indicating that there is no transcriptional regulation of amelogenins and collagen type I by PDGF in the cultured mouse molars. This suggests that PDGF does not function to induce the secretion of dentin or enamel extracellular matrices.

Since the DNA content of a normal, undividing somatic cell is constant, a change of total DNA reflects a change in cell number (Cunningham *et al.*, 1950). The cultured tooth organs showed no change in total DNA content among treatment groups, suggesting that there was no net increase or decrease in the total number of cells in response to PDGF-AA stimulation. Total RNA content, a second indicator of cell number, was also unchanged, which is consistent with this interpretation. A similar observation was made by Partanen *et al.* (1985) that PDGF did not increase cell number or affect morphogenesis of tooth germs grown in culture. The significant increase in tritiated thymidine incorporation in a background of stable total DNA and RNA content suggests that PDGF was increasing cell turnover.

A PDGF-AA concentration of 0.25 ng/ml was insufficient to stimulate a detectable mitogenic effect while high levels (25 ng/ml) may have induced a down regulation of the receptors that mediated the response at intermediate concentrations. Exposure to exogenous factor(s) can induce cells to down-regu-

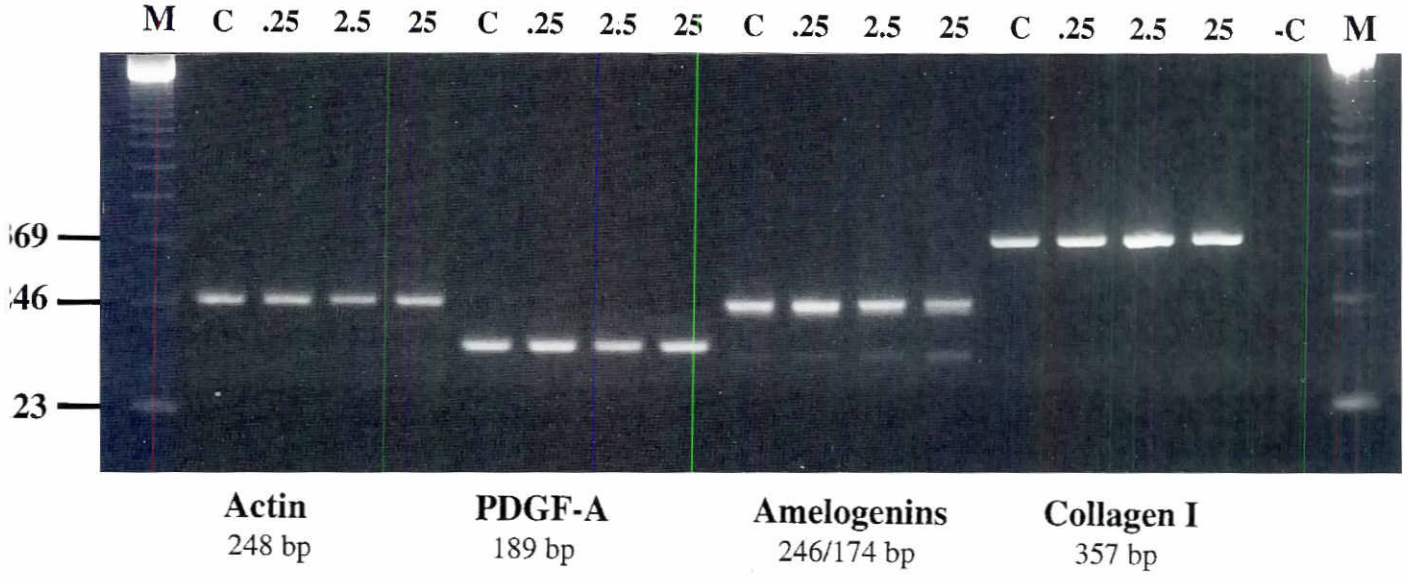

Fig. 4. RT-PCR amplification of dental matrix protein transcripts from PDGF treated mouse mandibular first and second molar explants (E15+7dc). *Lanes M: the 123 bp DNA marker bands; lanes C: control; lanes .25: PDGF-AA at concentration of 0.25 ng/ml; lanes 2.5: PDGF-AA at concentration of 2.5 ng/ml; lanes 25: PDGF-AA at concentration of 25 ng/ml; –C: reagent control. No detectable difference of the amplified transcripts PDGF-AA, amelogenins and collagen type I. ß-actin amplification serves as an internal control.*

late surface receptors specific for the exogenous factor or alternatively down regulate the factor itself. This negative feedback mechanism may protect cells against extreme changes in the activity of a receptor (see review by Oberg *et al.*, 1990). Such cellular responses are observed in cultured fibroblasts when stimulated by excessive epidermal growth factor (Carpenter and Cohen, 1976; Carpenter, 1987). Excess PDGF could decrease the production of PDGFR, increase the internalization of PDGF receptor or decrease the synthesis of endogenous PDGF-AA. Because supplementation of PDGF at progressively increasing concentrations did not alter the transcription of PDGF-AA in the molar explants we conclude that a reduction in PDGF receptor is a likely response to excess PDGF-AA.

A disadvantage of tooth organ culture is that the effects of exogenous growth factor are determined for the explant as a whole. If different tissues within the tooth organ react in opposite ways to a biological stimuli, no net effects are observed. To overcome this effect rat pulp tissue was grown in culture and stimulated with exogenous PDGF. Rat pulp was used because it can be more cleanly dissected away from other tissues and each subject provides 5-8 times more tissue than a mouse. We assume that rat and murine pulp cells are virtually identical due to the very close evolutionary relatedness of these two rodents of the subfamily Muridae.

PDGF is present *in vivo* in three dimeric forms: PDGF-AA, PDGF-AB and PDGF-BB. The effect of exogenous PDGF on cultured molars was limited to the AA isoform. PDGF-AA was selected because of its high affinity and specificity for the PDGFR-α, which is reported to be the dominant receptor type during mouse embryogenesis (Orr-Urtreger and Lonai, 1992). Therefore the effects of exogenous stimulation must be transduced through the PDGFR-αα dimer. It is possible that supplementation with other PDGF isoforms, which can also activate the PDGFR-ßß and PDGFR-αß, would produce different results.

However, this phenomenon was not observed when PDGF-AA and PDGF-BB were tested on cultured molar pulp cells. These two isoforms at a concentration of 25 ng/ml are potent mitogens in increasing pulp cell division *in vitro*. Although PDGF-BB binds to all three receptors and is reported to be a more potent mitogen than PDGF-AA, there was no significant difference observed in their mitogenic potential on pulp cells according to this series of *in vitro* experiments. Both PDGF-AA and PDGF-BB increased cell number *in vitro* 4 to 5 fold without increasing the total protein content of the cultured pulp cells. A possible explanation is that the cell division was not accompanied by significant amounts of cell growth.

These experimental findings document the association of PDGFs and their receptors within developing tooth organs, in particular the dental mesenchyme. The molecular machinery needed for a response to PDGF administration is present in differentiating odontoblasts and explanted tooth organs as well as molar pulp cells respond to PDGFs. Based on these findings, we are now using the rat incisor model to determine the effects of PDGF on odontoblast regeneration after injury.

Materials and Methods

Detection of PDGF-AA transcripts in mouse molars

Developing mouse mandibular first molars from embryonic day 14 (E14) through post-natal day 2 (PN2) were extracted; molars from E15 to newborn (NB) were treated with undiluted Dispase at 37°C for 15 min (Boehringer Mannheim, Indianapolis, USA), the epithelial and mesenchymal tissues separated, and total RNA from the dental epithelium and ectomesenchyme was isolated. The RNA extraction, reverse transcription and polymerase chain reaction were performed according to the published protocol (Hu *et al.*, 1992). The presence or absence of messages for PDGF-AA in each RNA preparation was determined by RT-PCR using a primer set specific for PDGF-AA, 189 bp product using primers 5'-AGGAAGCCATTCCTGCA and 5'-CTTGACACTGCGGTG-

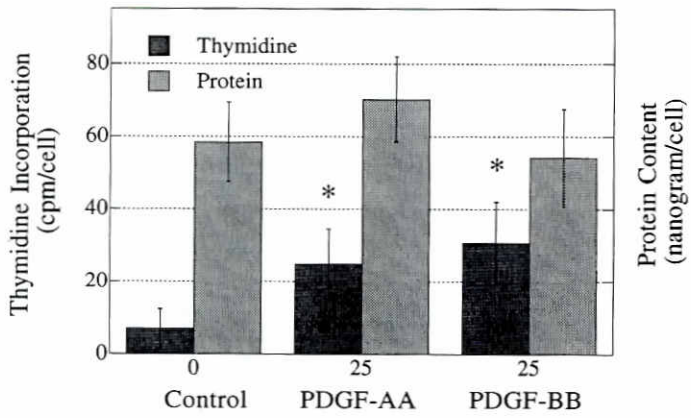

Fig. 5. Changes in thymidine incorporation and protein content in rat pulp cell cultures following exogenous PDGF-AA or PDGF-BB administration. *Asterisk denotes statistic significance at p<0.05. PDGFs at concentration of 25 ng/ml increased cell proliferation but not total protein accumulation in these cultured pulp cells.*

GTG (Orr-Urtreger and Lonai, 1992); the amplification of ß-actin, 248 bp product using primers 5'-CATCGTGGGCCGCTCTAGGCACCA and 5'-GGGGGACTTGGGATTCCGGTTGGC (Hu *et al.*, 1992) was the internal control. The amplification products were separated on a 4% agarose gel, visualized by ethidium bromide staining, and photographed. Parallel amplification of ß-actin from each RNA preparation demonstrated the effectiveness of the RNA extraction.

Immunolocalization of PDGF and PDGFR in mouse molars

Rabbit anti-human polyclonal antibodies raised against PDGF-A/B and PDGFR-α/ß (UBI, Lake Placid, NY, USA) with cross-reactivity in rodent tissues were used for immunolocalization. The antibody against PDGF-A/B recognizes specific epitopes in PDGF A chain and B chain thus it can detect all three isoforms PDGF-AA, -BB and -AB. The antibody against PDGFR-α/ß recognizes the epitopes in PDGFR-α and PDGFR-ß so it can detect the PDGFR-αα, -ßß as well as -αß. Developing mouse first molars were extracted from E14 through PN1, fixed in buffered formalin, embedded in paraffin, sectioned to the thickness of 5 µm, and immunostained with HSP kit (Zymed, San Francisco, USA) according to the published protocol (Hu *et al.*, 1992). The mouse molar sections were incubated with the PDGF-A/B antibody at concentration of 1:150 or PDGFR-α/ß antibody at 1:100 for 1 h at room temperature. Negative control samples were processed through the same procedure but incubated with PBS instead of primary antibodies.

Effect of PDGF-AA on cell proliferation in developing mouse molars

Embryonic day 15 mouse mandibular first and second molars (6 explants/culture) were maintained in a serumless, chemically-defined culture system (Bringas *et al.*, 1987; Evans *et al.*, 1988) supplemented with 0.0, 0.25, 2.5 or 25 ng/ml PDGF-AA (USB, Cleveland, USA), with media changes every 48 h. On culture day 7 (7 dc) ^{3}H-thymidine at 20 µCi/ml (NEN, Boston, USA) was added and the cultures incubated for 4 h followed by a 1 h cold thymidine chase. Chemical assays were performed to measure the cell proliferation (^{3}H-thymidine incorporation), total DNA, RNA and protein content of the explanted molars (Lowry *et al.*, 1951; Keleti, 1974). The experiment was repeated 3 times to measure statistical significance.

Effect of PDGF-AA on dental matrix protein production

To investigate the effect of exogenous PDGF-AA on mRNA levels of PDGF-AA, amelogenins, and type I collagen, polymerase chain reaction was performed with standardized quantity of samples for 25 cycles. Embryonic day 15 mouse mandibular first and second molars (6 explants/culture) were maintained in a serumless, chemically-defined culture system (Bringas *et al.*, 1987; Evans *et al.*, 1988) supplemented with 0.0, 0.25, 2.5 or 25 ng/ml PDGF-AA (USB, Cleveland, USA), with media changes every 48 h. The cultured tooth organ were collected on day 7, frozen immediately and used for amplification of (1) ß-actin, (2) PDGF-AA, (3) amelogenin, 5'-TGGTGTTGGG ATTGGAGTCATGG and 5'-GGTGAAGTTTCCCCAT/CTCGTGG (246- and 174-bp) (Lau *et al.*, 1992); and (4) type I collagen, 5'-TTCTGGATCAAGTGGTGAAC and 5'-GTTCCACTGTCCCGTATTT (357-bp) (Monson *et al.*, 1982). In each preparation the amount of RNA used for amplification is less than 1 ng which was pooled from six molar explants.

Effect of PDGF-AA on pulp cell proliferation

Postnatal day 6 Sprague Dawley rats were used to provide pulp tissues. Pulp cell culture was established according to the published protocol (Mosmann, 1983; Andrews *et al.*, 1993). Upon decapitation, all molars of the rat were removed and placed in Hank's Balanced Salt Solution (Life Technologies, Inc., Grand Island, NY, USA). The pulp tissue was dissected into small pieces, digested with 0.25% Trypsin/EDTA (Life Technologies, Inc. Grand Island, NY, USA) at 37°C for 15 min, rinsed, centrifuged and resuspended with RPMI 1640 with ascorbic acid, antibiotics and 10% fetal bovine serum (Life Technologies, Inc. Grand Island, NY, USA). Pulp cells at the amount of $1x10^4$ were plated onto the 75 ml vented flasks and received fresh medium every 48 h. Attachment of the cells occurred in 1-2 days and confluence was achieved in 6-7 days. First passage was performed upon confluence. When confluence was achieved following the passage, cells were treated with serum free RPMI for 24 h succeeded by 1) RPMI with 10% fetal bovine serum, 2) RPMI with 25 ng/ml PDGF-AA and 10% fetal bovine serum, or 3) RPMI with 25 ng/ml PDGF-BB and 10% fetal bovine serum for 24 h. The concentration of PDGFs was determined based on multiple *in vivo* and *in vitro* studies (Knighton *et al.*, 1990; Nakashima, 1992; Robson *et al.*, 1992). At the end of the incubation, the cultures were incubated with ^{3}H-thymidine (NEN, Boston, USA) at 100 µCi/5 ml medium for 4 h followed by a 1 h cold thymidine chase. Chemical assays were performed to measure the cell proliferation (^{3}H-thymidine incorporation), total DNA and protein content of the cultured pulp cells (Lowry *et al.*, 1951; Keleti, 1974). The amount of thymidine incorporation was divided by the cell number obtained from dividing the total DNA content with the DNA content of a single rat cell, $6.5x10^3$ ng/diploid cell. The experiment was repeated 3 times to measure statistical significance.

Statistic analyses

All studies were done in triplicate. Data were analyzed by using one-way analysis of variance and Student's *t* test, taking the confidence level at p<0.05 (Epistat statistic program).

Acknowledgments

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