Articles

Isolation and Partial Characterization of Mitogenic Factors from Cementum†

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ABSTRACT: Cementum is the mineralized structure through which soft connective tissues are attached to the teeth. It is a unique calcified tissue characterized by a low metabolic turnover, lack of blood supply, and presence of very few cells. However, it contains substances that influence the biological activities of fibroblasts of adjacent soft tissues. We have partially characterized cementum proteins that have mitogenic activity toward fibroblasts. Cementum was harvested from bovine teeth, and mitogenic factors were extracted in 0.5 M CH_3COOH. Heparin–Sepharose chromatography separated the mitogenic activity into a major and a minor fraction eluted by 0.5 and 2.0 M NaCl, respectively. The distribution of cementum mitogens in heparin–Sepharose fractions was different from that of alveolar bone and other bones. The cementum mitogenic factor eluting with 2.0 M NaCl from a heparin–Sepharose column was shown to be basic fibroblast growth factor (bFGF) on the basis of inhibition by anti-bFGF antibody and Western blots. The 0.5 M NaCl fraction was purified by HPLC with use of a combination of a DEAE-3W column followed by TSK-250 and C18 columns. $NaDodSO_4$–polyacrylamide gel electrophoresis revealed that the purified fraction contained two protein bands with M_r 22 000 and 19 000, and mitogenic activity was associated with the M_r 22 000 species. The activity of this mitogen, designated as CGF, was potentiated by small quantities of plasma-derived serum or epidermal growth factor. It was heat resistant, but was destroyed by reduction. Assays of CGF preparations revealed that they contained no detectable platelet-derived growth factor. We conclude that cementum has a characteristic distribution of mitogenic factors and that CGF is the major cementum mitogen. Even though the CGF resembles PDGF in some properties, it appears to be a different mitogen.

Mineralized matrix of osseous tissues has been shown to sequester a variety of growth regulatory molecules such as acidic and basic FGF,[1] PDGF, TGF-β, and osteoinductive factor (Hauschka et al., 1986; Katz & Reddi, 1988; Luyten et al., 1989; Mayer & Kukoschke, 1989; Wozney et al., 1988). These substances are thought to play a key role in the turnover, growth, and development of bones and cells they harbor and in the healing of fracture wounds. However, unlike other mineralized structures, cementum has a low metabolic turnover, it contains very few cells, and it does not have a blood supply, lymph drainage, or innervation (Schluger et al., 1990). Nevertheless, extracts of cementum have been shown to promote the growth, attachment, and movement of fibroblasts and their synthesis (Miki et al., 1987; McAllister et al., 1990; Somerman et al., 1987b; 1989; Nishimura et al., 1989). The role played by these substances is not clear even though several observations indicate that cementum may affect the formation and regeneration of adjacent connective tissues that are attached onto its surface, especially the periodontal ligament. For example, the fibroblast attachment, growth, and chemotactic activities found in cementum are not detectable in the neighboring connective tissues gingiva and periodontal ligament or in dentin (Nishimura et al., 1989; McAllister et al., 1990; Somerman et al., 1987b, 1989; Miki et al, 1987). Cementum affected by disease is unable to support fibroblast attachment or growth, and reattachment of connective tissue does not occur to cementum damaged by chronic inflammation (Fernyhough & Page, 1983; Wirthlin & Hancock, 1980). Thus, the cementum appears to represent a unique model in which to study the mechanisms by which a calcified tissue may influence the structure, function, formation, and regeneration of neighboring soft connective tissues. In this paper we report the separation of mitogenic factors present in cementum, whose distribution appears to be different from other mineralized tissues. We also report partial characterization of two cementum mitogens, one of which is bFGF and another that appears to be a different molecule.

Experimental Procedures

Materials. Bovine cementum was obtained from the mandibular molars of two-year-old animals and stored frozen at −20 °C until use. Heparin–Sepharose CL6B and CM-Sephadex C-50 were products of Pharmacia-LKB Biotechnology, Uppsala, Sweden. Proteinase inhibitors phenylmethanesulfonyl fluoride, pepstatin A, and leupeptin were purchased from Boeringer Mannheim Biochemical, Indianapolis, IN, and *N*-ethylmaleimide was obtained from Sigma Chemical Co. St. Louis, MO. Polyclonal rabbit antibody to bFGF was a generous gift from Dr. Andrew Baird, Whittier Institute for Diabetes and Endocrinology, San Diego, CA, and bFGF was obtained from Collaborative Research Inc. Acidic FGF and anti-aFGF antibody were purchased from R and D systems, Minneapolis, MN. Goat anti-rabbit IgG was the product of Miles Scientific, Naperville, IL; ultrafiltration materials and Centriprep 10 were obtained from Amicon,

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[1] Abbreviations: a- and bFGF, acidic and basic fibroblast growth factor; CGF, cementum-derived growth factor; DV-medium, Dulbecco–Vogt medium; EGF, epidermal growth factor; GFU, growth factor units; IGF, insulin-like growth factors; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β.

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Danvers, MA, and HPLC columns were from Bio-Rad, Richmond, CA. Immobilon-P PVDF membrane was the product of Millipore Corp., Bedford, MA. [*methyl*-^{3}H]Thymidine was purchased from New England Nuclear, Boston, MA, and Ecolite 4 was from ICN Biomedicals, Irvine, CA. All other chemicals were obtained from other manufacturers and were of ultrapure or comparable grade.

Methods. Extraction of Cementum. Teeth were thoroughly cleaned, and adhering periodontal ligament and other connective tissues were mechanically removed. Cementum was then scraped with curettes into 50 mM Tris-HCl, pH 7.5, containing 25 mM EDTA, 1 mM each phenylmethanesulfonyl fluoride and *N*-ethylmaleimide, and 1 μg/mL each pepstatin A and leupeptin (Miki et al., 1987). It was then dialyzed against several changes of 0.5 M CH_3COOH containing the proteinase inhibitors at 4 °C for 3–4 days. The retentate contained >75% of the mitogenic activity present in cementum (Miki et al., 1987). This was concentrated by ultrafiltration through an Amicon YM10 membrane and dialyzed against 10 mM Tris-HCl buffer/50 mM NaCl, pH 7.4. All the subsequent manipulations were done at 4 °C.

Heparin–Sepharose Chromatography. Proteins were loaded on a column (0.9 × 20 cm) in 10 mM Tris-HCl buffer/50 mM NaCl, pH 7.5, and washed with the buffer to remove unbound proteins. The column was then eluted as specified, either stepwise by 0.2, 0.5, and 2.0 M NaCl or with a 0–2.0 M linear NaCl gradient. Fractions were collected and monitored for proteins by absorbance at 230 nM and assayed for mitogenic activity.

HPLC of Heparin–Sepharose 0.5 M NaCl (HS–0.5) Fraction. The HS–0.5 fractions were dialyzed against 10 mM Tris-HCl, pH 7.0, buffer and then loaded on a Bio-Sil TSK DEAE-3SW column. The column was washed with buffer to remove unbound proteins and then eluted with a multilinear NaCl gradient at a flow rate of 0.5 mL/min. One-minute fractions were collected and monitored at 230 and 280 nM for proteins and assayed for mitogenic activity. Fractions with mitogenic activity eluted by 0.25–0.4 M NaCl were pooled and subjected to molecular sieve chromatography through a Bio-Sil TSK-250 column. Separation was achieved in 10 mM Tris-HCl/150 mM NaCl buffer, pH 7.0, at a flow rate of 0.5 mL/min. Active fractions from this column were collected and reverse-phase chromatography was performed with use of a μBONDPACK C_{18} column. The latter was equilibrated in 0.1% trifluoroacetic acid and eluted with a multilinear gradient of acetonitrile at a flow rate of 0.5 mL/min. One minute fractions were collected.

Assay for mitogenic activity was performed with use of human gingival fibroblasts as described previously (Miki et al., 1987). Briefly, subconfluent fibroblasts were made quiescent by serum starvation for 48 h and activated by mitogen alone or mitogen plus 1% plasma-derived serum. After 22 h, medium was replaced by fresh serum-free medium containing 2 μCi/mL [^{3}H]thymidine. Four hours later, medium was removed and cells were washed three times with ice-cold phosphate-buffered saline pH 7.2, solubilized in 0.05 N NaOH and counted in Ecolite 4. The mitogenic activity is expressed as counts per minute of [^{3}H]thymidine uptake (DNA synthesis) or as growth factor units (GFU), where one unit is equal to the [^{3}H]thymidine uptake induced by 10% fetal bovine serum under same conditions.

$NaDodSO_4$ polyacrylamide gel electrophoresis was performed without reduction on 12.5% slabs as described previously (Narayanan & Page, 1983), and proteins were located by Coomassie blue or silver staining (Oakley et al., 1980). For determination of activity, proteins were eluted in 1 M CH_3COOH containing 0.1% $NaDodSO_4$ and concentrated by acetone precipitation (Raines & Ross, 1982).

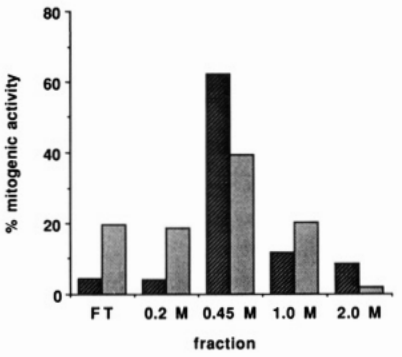

FIGURE 1: Distribution of mitogenic activity of cementum and alveolar bone extracts in heparin–Sepharose fractions. A total of 10 g of cementum and 80 g of alveolar bone from which periosteum and marrow have previously been removed were extracted. The extracts were dialyzed against 10 mM Tris-HCl pH 7.4/50 mM NaCl and loaded on a heparin–Sepharose CL-6B column (1.5 × 11 cm) at a flow rate of 20 mL/h. The column was then washed with the equilibration buffer and eluted stepwise with NaCl as indicated. Hatched bars represent cementum and stippled bars the alveolar bone. FT: flow-through fraction.

Immunoblot. After electrophoresis, proteins were electroblotted onto a PVDF membrane with use of a Bio-Rad transblot cell. The membrane was blocked with 3% bovine serum albumin and incubated with a polyclonal anti-bFGF antibody. Bound antibody was visualized by incubation with peroxidase labeled goat anti-rabbit IgG, peroxidase–antiperoxidase, and diaminobenzidine.

Protein concentrations were determined with a Bio-Rad protein assay kit by the Bradford assay (Bradford, 1976) with bovine serum albumin used as standard.

RESULTS

Heparin–Sepharose Separation of Cementum and Alveolar Bone Mitogens. Previously we showed that 0.5 M CH_3COOH extracts of human and bovine cementum were mitogenic for human fibroblasts whereas extracts of dentin, which is a calcified structure contiguous with cementum, had very little activity (Miki et al., 1987). In contrast, extracts of alveolar bone, which houses the teeth, were mitogenically active. Approximately 7 GFU of mitogenic activity/g of wet weight of alveolar bone was obtained, while cementum contained 5–10 GFU/g. In order to determine whether both cementum and alveolar bone contain the same mitogenic factors, extracts from both tissues were subjected to chromatography on a heparin–Sepharose column, which separates several growth factors on the basis of affinity to heparin (Shing et al., 1984; Hauschka et al., 1986; Gospodarowicz, 1987; Quinkler et al., 1989). From Figure 1 it is seen that the distribution of mitogenic activity in the various NaCl fractions was different for both tissues. In cementum peparations, the 0.45 M NaCl eluate contained the majority (68%) of the loaded mitogenic activity. Less than 10% was present in the flow-through plus 0.2 M NaCl fractions, while 1.0 and 2.0 M NaCl fractoins had 12 and 9%, respectively (Figure 1). The 0.45 NaCl fraction of the alveolar bone also contained the major portion of the mitogenic activity; however, it was relatively less than in cementum (39 vs 68%). A considerably greater proportion of the alveolar bone mitogens did not bind or bound weakly to the heparin (20 and 19% of the total activity was present in flow-through and 0.2 M NaCl fractions, respectively). More activity was also present in the 1.0 M NaCl fraction, which contained 20% of the total (Figure 1).

Table I: Effect of Anti-bFGF Antibody on Mitogenic Activity of Cementum HS–2.0 Fraction

addition	antibody[a]	DNA synthesis cpm × 10^{-3} [b]	%
none	–	1.2 ± 0.2	
HS–2.0	–	52.6 ± 3.2	100
HS–2.0	+	14.1 ± 2.2	25
pFGF[c]	–	29.2 ± 2.6	100
bFGF[c]	+	10.3 ± 1.0	33

[a] Added at 1:1000 dilution. [b] [^{3}H]Thymidine uptake, mean ± SD of triplicates. [c] 1 ng/mL.

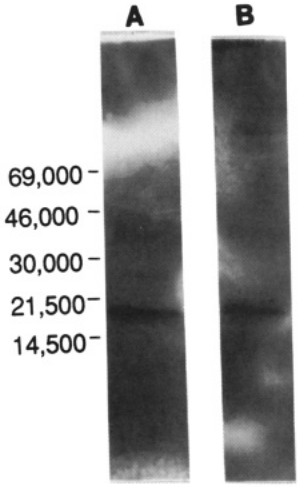

FIGURE 2: Western blot of cementum HS–2.0 fraction using anti-bFGF antibody. A: bovine pituitary FGF; B: cementum HS–2.0. The migration of some molecular weight markers is indicated.

Characterization of 2.0 and 1.0 M NaCl Heparin–Sepharose Fractions of Cementum. Since bFGF elutes from heparin–Sepharose at high salt concentrations (Shing et al., 1984) and it has been identified in mineralized matrix (Hauschka et al., 1986), the 2.0 M NaCl eluate from heparin–Sepharose (HS–2.0) was examined for the presence of bFGF by utilizing a polyclonal anti-bFGF antibody obtained from Dr. Andrew Baird. The anti-FGF antibody inhibited >70% of the mitogenic activity in this fraction (Table I). Western blots using this antibody revealed a strong immunoreactive band at M_r 18 000 in the HS–2.0 comparable to a standard of bovine pituitary bFGF (Figure 2). The mitogenic activity in the HS–2.0 represented <10% of the crude extract, while it represented <1% of the protein. We also attempted to characterize the mitogen in the 1.0 M NaCl fraction. Because the 1.0 M eluate is likely to contain aFGF (Gospodarowicz, 1987), presence of this mitogen was examined by Western blots and inhibition in mitogenic assays by use of anti-aFGF antibody. However, even though significant mitogenic activity was recovered in Figure 1, other preparations did not yield enough material to be detected in these assays.

Characterization of Heparin–Sepharose 0.5 M NaCl Fraction. PDGF is eluted from heparin columns by 0.45–0.5 M NaCl (Raines & Ross, 1982; Hauschka et al., 1986); therefore, the column was eluted with 0.5 M NaCl and cementum fractions were assayed for this growth factor. However, no PDGF could be detected in this fraction (see below). Therefore, this fraction, which represented 55–70% of all cementum mitogenic activity in different experiments, was further purified. First it was subjected to ion-exchange chromatography by HPLC with using a Bio-Sil TSK DEAE-3SW column (Figure 3). This procedure separated a significant portion of proteins from mitogenic activity, most of which was recovered between 0.25 and 0.4 M NaCl (peak B, Figure 3). The peak B fractions containing mitogenic activity were pooled, concentrated, and fractionated on a HPLC Bio-Sil TSK-250 column. The proteins resolved into three major peaks, while most of the mitogenic activity eluted as a broad peak between M_r 18 000 and 35 000 (not shown) with 3–4-fold purification (Table II). The active fractions were pooled and separated on a μBONDPACK C_{18} reverse-phase HPLC column with an acetonitrile gradient (Figure 4). A single peak of mitogenic activity was recovered as a trailing end of the major protein peak at 36% acetonitrile concentration. This fraction was designated as CGF and it represented

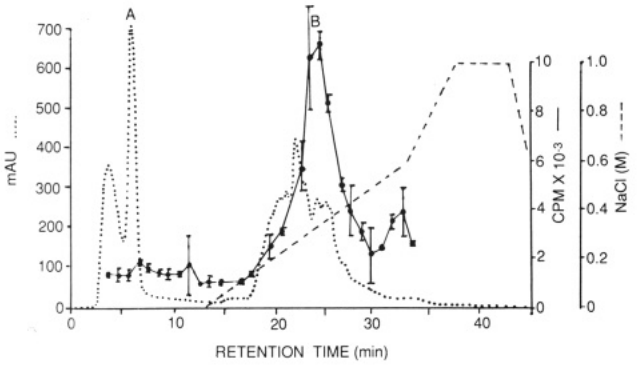

FIGURE 3: DEAE-HPLC of HS–0.5 M NaCl fraction of cementum. The 0.5 M eluate obtained from the heparin–Sepharose column (Figure 1) was dialyzed against 10 mM Tris-HCl buffer, pH 7.0, and applied on a DEAE-3SW HPLC column equilibrated with the same buffer. The column was eluted with a multilinear gradient of NaCl at a flow rate of 0.5 mL/min, and 0.5-mL fractions were collected. Fractions were diluted 10-fold in 1% BSA and PBS and 5 μL of the aliquots were assayed for mitogenic activity. Key: (···) absorbance; (---) NaCl concentration; (—) mitogenic activity. Mean ± SD of triplicate assays is shown.

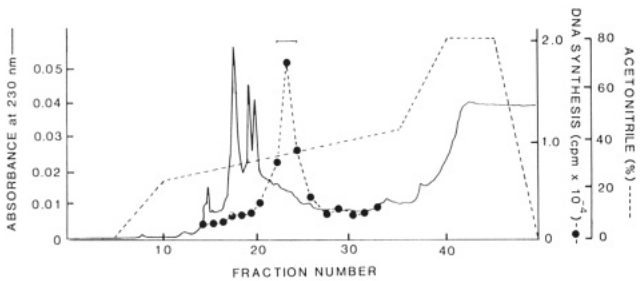

FIGURE 4: C_{18} reverse-phase HPLC of the DEAE-B fraction from Figure 3. After gel filtration through TSK-250 columns, fractions of DEAE-B were applied on a C_{18} column equilibrated with 0.1% CF_3COOH. Proteins were eluted with a multilinear gradient of CH_3CN in 0.1% CF_3COOH at a flow rate of 0.5 mL/min; fractions of 0.5 mL were collected and aliquots were assayed for mitogenic activity. Key: (—) absorbance; (---) percent acetonitrile; (●) mitogenic activity.

Table II: Purification Summary for Growth Factor from Cementum (CGF) from One Representative Preparation

fraction	μg of protein	sp act.[a]	purification (x-fold)	yield (%)
acid extract	110000	11	1	100
heparin–Sepharose, 0.5	2933	254	23	61
DEAE-HPLC	288	1263	115	30
molecular sieve HPLC	65	5061	460	27
C_{18} reverse-phase	2.3[b]	93817	8529	18

[a] GFU/mg, of protein; one unit of GFU is equal to the [^{3}H]thymidine incorporation induced by 10% fetal bovine serum. Assays were performed in a total volume of 0.5 mL of serum-free DV medium containing 5 μL of plasma-derived serum (1% concentration) plus CGF fractions, or 50 μL (10%) of fetal bovine serum. [b] Protein was determined from the area of the peaks in HPLC.

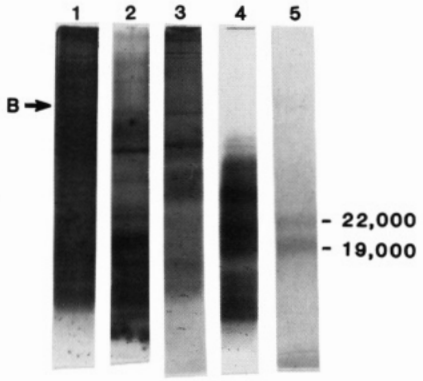

FIGURE 5: $NaDodSO_4$–polyacrylamide gel (10%) electrophoresis of cementum mitogenic factor. The electrophoresis was performed on samples without reduction under nonreducing conditions. Lane 1: crude extracts. Lane 2: HS–0.5 fraction. Lane 3: DEAE-B fraction. Lane 4: molecular sieve chromatography fraction. Lane 5: after C_{18} reverse-phase chromatography. The protein bands migrating with M_r 22 000 and 19 000 are shown. B: migration of bovine serum albumin.

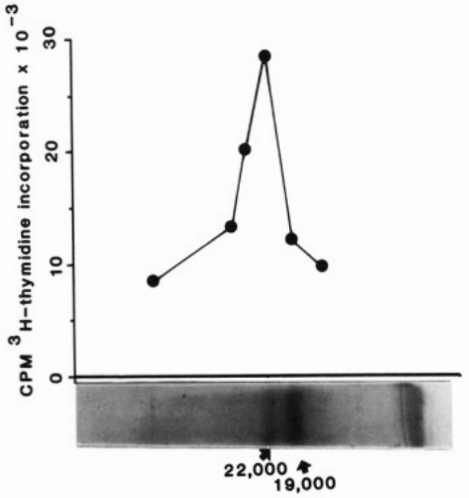

FIGURE 6: Elution of mitogen from $NaDodSO_4$–polyacrylamide gel. After electrophoresis under nonreducing conditions, the gel was cut into slices, proteins, were eluted, and mitogenic activity was assayed as described in the Experimental Procedures section.

8500-fold purification relative to the crude extract (Table II, 3000–12 000-fold in various preparations). The recovery of mitogenic activity was 18% relative to the crude starting material, even though protein contained in this fraction represented 0.002% of the starting material (Table II).

Figure 5 shows a silver-stained $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of CGF fractions at various stages of purification. Two protein bands with M_r 22 000 and 19 000 were present in the final C_{18} column fraction. In order to identify which of these bands has mitogenic activity, proteins from corresponding unstained gels were eluted and mitogenic activity was determined. The results showed that mitogenic activity was associated with only the M_r 22 000 protein band (Figure 6).

Properties of the CGF. Approximately 12–47 ng/mL of the C_{18} column fraction was required for optimal mitogenic stimulation of fibroblasts. It was mitogenic alone, but its activity at suboptimal concentrations was potentiated by EGF, 1% plasma-derived serum (Table III), or 0.2% fetal bovine serum (not shown). The CGF activity was resistant to heating at 60 °C for 20 min; however it was almost completely destroyed by trypsin and dithiothreitol (Table IV). These properties are characteristic of PDGF and therefore the C_{18} preparation as well as the DEAE fractions was assayed for

Table III: Synergism of Mitogenic Activity of CGF with Other Factors.

addition[a]	[^{3}H]thymidine uptake (cpm × 10^{-3} [b])	% maximum
EGF, 5 ng/mL	3.6 ± 0.5	7
1% plasma-derived serum	8.0 ± 0.7	15
CGF	9.8 ± 0.5	19
CGF + 5 ng/mL EGF	20.8 ± 1.8	40
CGF + 1% plasma-derived serum	50.9 ± 3.6	97
PDGF[a]	10.5 ± 0.6	20
PDGF + 5 ng/mL EGF	24.2 ± 1.6	46
PDGF + 1% plasma-derived serum	40.0 ± 3.2	76
10% fetal bovine serum	52.4 ± 7.0	100

[a] CGF and human platelet PDGF-AB were added at 20 ng and 1 ng/mL concentration; these concentrations induced 20% of optimal mitogenic activity. [b] mean ± SD of triplicates.

Table IV: Properties of Cementum Mitogenic Factor[a]

treatment[b]	% activity recovered[c]
none	100
heat	93 ± 11
trypsin	21 ± 9
DTT	7 ± 9

[a] CGF fraction after C_{18} reverse-phase HPLC. [b] 100 μL of C_{18} fraction containing 250 ng of protein was either heated at 60 °C for 30 min, digested with 5 μg of trypsin for 1 h at 37 °C, or treated with 0.01 M dithiothreitol and 0.025 M iodoacetamide as described by Raines and Ross (1982). Treated and untreated samples were assayed for mitogenic activity at a 20 ng/mL concentration, simultaneously under same conditions. Recoveries are expressed relative to respective untreated controls. [c] mean ± SD of three experiments.

PDGF by the radioreceptor assay (Bowen-Pope & Ross, 1985), by ELISA using a polyclonal antibody (which recognizes all forms of PDGF; Shimokado, et al., 1985; Raines et al., 1989), and monoclonal antibodies specific to A- and B-chains (Ross et al., 1990; Hart et al., 1990). However, no PDGF was detected by these approaches in preparations containing at least 10-fold higher amounts of mitogenic activity (as determined by mitogenic activity of the purified fractions as compared with a PDGF standard curve; 5 ng/mL PDGF is required for optimum stimulation of gingival fibroblasts). The preparations were also acidified and fractionated to remove possible binding proteins (Raines & Ross, 1985, 1987), but no PDGF was detected. Antibodies prepared against CGF also had no effect on any of the dimeric forms of PDGF.

Discussion

Our data show that cementum, similar to other calcified tissues, contains several mitogenic factors that can be separated on the basis of differences in affinity to heparin. The cementum contains mitogenic activity for fibroblasts equivalent to 25–50 ng of PDGF/g of wet tissue, which is comparable to 35 ng/g in alveolar bone. However, the distribution of mitogens in cementum appears to be different from in other mineralized tissues. For instance, while growth factors having high affinity to heparin (a- and bFGF) account for greater than 60% in other mineralized tissues (Hauschka et al., 1986), they form only ~10% in cementum. Even in alveolar bone, a calcified tissue in close proximity to cementum, a- and bFGF together account for ~20% of the total mitogenic activity. Furthermore, approximately 40% of the mitogenic activity of alveolar bone does not bind or binds only weakly to heparin while these fractions form ~10% in cementum. These fractions, which are likely to contain EGF and IGF (Hauschka et al., 1986), were not characterized.

The cementum mitogen in the HS-2.0 fraction was characterized as bFGF on the basis of strong affinity to heparin, inhibition by anti-bFGF antibody, and Western blots. The small amount of mitogenic activity eluted by 1.0 M NaCl from heparin-Sepharose columns is likely to represent aFGF (Gospodarowicz, 1987); however, this could not be confirmed because we were unable to obtain sufficient material.

The CGF characterized in this paper is the major cementum mitogen. It has moderate affinity for heparin from which it is dissociated by 0.45–0.5 M NaCl. The elution properties of CGF from heparin columns, synergism with EGF and serum factors, heat resistance, and susceptibility to reduction all resemble those of PDGF. However, we could not detect PDGF in purified as well as crude preparations by procedures capable of detecting both A- and B-chains of PDGF. PDGF was also not detected in preparations obtained from human cementum (data not shown), showing that the inability to detect PDGF is not due to bovine vs human species difference. A monoclonal antibody against bovine CGF, which inhibits human CGF action, does not recognize human PDGF (Nakae et al., manuscript in preparation). PDGF and fetal bovine serum (which contains PDGF) reduce EGF binding to fibroblast membranes (Raines et al., 1990); however, CGF has no effect.[2] These data together indicate that the CGF is a different polypeptide from PDGF. Other properties of CGF also support this possibility. Most significantly, its molecular size of M_r 22000 is different from human and porcine PDGF species (M_r 28000–31000; Raines et al., 1990; Stroobant & Waterfield, 1984). The electrophoretic mobility of CGF is also not significantly affected by reduction[3] while PDGF is reduced to subunits with M_r 14400, 16000 and 17500 (Raines & Ross, 1982). We do not believe that CGF is a degradation product of PDGF because precautions were taken to prevent proteolysis and no cross-reactivity was observed in ELISA assays with three different anti-PDGF antibodies. Additionally, in molecular sieve and C_{18} columns run under identical conditions, authentic human PDGF (A–B dimer) eluted differently from CGF (data not shown). Finally, assay procedures to remove binding proteins that might prevent PDGF detection also failed to detect PDGF in CGF preparations, even at 10 times the optimal concentration. These data together indicate that CGF is distinct from PDGF. They, however, do not rule out the possibility that the cementum mitogen may represent a member of proteins closely related to PDGF. The CGF does not appear to be TGF-β because CGF does not stimulate collagen and protein synthesis, while TGF-β does[4] (Miki et al., 1987; Narayanan et al., 1989). It also appears to be unrelated to osteogenin, which has different solubility properties (Katz & Reddi, 1988; Vukicevic et al., 1990). Assays to detect osteogenin in CGF preparations were also negative.[5] Additional characterization of CGF is in progress to determine the biochemical nature of the CGF and its relationship to PDGF and other growth factors.

In addition to CGF and FGF, the cementum also contains mitogens in fractions that do not bind or that bind weakly to heparin. These fractions, which are likely to contain EGF and IGF (Hauschka et al., 1986), were not characterized. Even though we could not detect their presence, others have reported that cementum contains TGF-β-like and bone-inductive activities (Somerman et al., 1987a; Kawai & Urist, 1989). Thus, cementum appears to contain a battery of biologically active molecules. However, the origin of these substances in cementum is not known. One possibility is sequestration from blood; however, this tissue has no direct blood supply. Osteoblasts and endothelial cells have been shown to produce bFGF and other growth factors in bone, however the cementoblast is the cell type present in cementum. The cementoblasts are present in very small numbers in cementum, and whether these cells are capable of producing cementum mitogens, which are present in concentrations comparable to those in other bones, is not known.

It is unclear from our studies whether the mitogenic activity we have characterized is intracellular or is associated with cementum matrix, even though bFGf has been described as associated with matrix in vivo in a number of situations. Immunolocalization studies will help to identify the cellular source and localization of the mitogens in cementum. The biological role of cementum growth factors is also not clear. Cementum consists of cellular and acellular portions; thus, one role of cementum mitogens may be to regulate the cells present in cementum. This regulation could be autocrine and/or paracrine. However, this must be a limited role because cementum has very little turnover and it undergoes very little remodeling. Interestingly, cementum contains additional substances that promote fibroblast attachment and chemotaxis, and these activities are low or not detectable in the adjacent dentin and connective tissues (Nishimura et al., 1989; McAllister et al., 1990; Miki et al., 1987; Somerman et al., 1987b, 1989). These observations indicate that cementum may be capable of regulating the metabolism and turnover of surrounding tissues. More importantly, it may participate in the formation and regeneration of periodontal ligament, a soft tissue that is responsible for anchoring the teeth. The attachment of this connective tissue to tooth surfaces is lost in chronic inflammation, and it is rarely restored. Indeed, restoration of attachment is a major therapeutic challenge. Because cementum exposed to the contents of periodontal pockets is altered structurally and diseased cementum does not support fibroblast attachment or growth (Pitaru & Melcher, 1983; Fernyhough & Page, 1983; Aleo et al., 1975; Cogen et al., 1984; Olson et al., 1985), it appears likely that the neutralization of cementum factors is one reason why connective tissue does not form on diseased tooth surfaces. A study of the localization and interaction between cementum components with adjacent tissues is therefore necessary to understand the mechanisms of connective tissue regeneration and reattachment to diseased teeth, and, more importantly, this system can serve as a unique model to examine how calcified structures influence neighboring tissues.

[2] K. Yonemura and A. S. Narayanan, unpublished data.

[3] The M_r 22000 band cut from gels previously run without reduction also migrates corresponding to M_r 22000 upon reelectrophoresis under reducing conditions (K. Yonemura and A. S. Narayanan, unpublished data).

[4] Addition of cementum extracts to fibroblasts does not cause stimulation of collagen and protein synthesis while that of TGF-β does, and at 10 ng/mL concentration TGF-β have very little mitogenic activity towards fibroblasts (Miki et al., 1987; Narayanan et al., 1989).

[5] Osteogenin is not mitogenic to human gingival fibroblasts (Miki et al., 1987). In addition, assays were performed by Dr. Hari Reddi at NIDR, Bethesda, MD, to detect osteogenin, but the results were negative.

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