Mitogenic Signaling Mechanisms of Human Cementum-derived Growth Factor*

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Cementum-derived growth factor (CGF) is a M, 23,000 protein, which is sequestered in the mineralized matrix of tooth cementum. We have investigated the mitogenic signaling reactions induced by CGF using quiescent human gingival fibroblasts as target cells. Cells activated with CGF were compared with those treated with CGF plus epidermal growth factor (EGF) and other growth factors. CGF caused a transient increase in cytoplasmic Ca²⁺ concentration, and this was accompanied by enhancement of membrane protein kinase C activity, myelin basic protein and S₆ kinase activities, inositol phosphate levels, and activation of c-fos and jun-B gene expression. Membranes obtained from cells activated with CGF contained several protein bands, which cross-reacted with anti-phosphotyrosine antibody; however, proteins corresponding to a putative phosphorylated CGF receptor were not detected. DNA synthesis induced by CGF was inhibited by 65% in cells treated with pertussis toxin but only 25–29% in cultures exposed to H7 or 12-O-tetradecanoylphorbol-13-acetate; these values were different from those obtained when EGF, PDGF, or fetal bovine serum were used as mitogens. CGF and TGF-β, but not EGF, caused an increase of PDGF-A chain mRNA expression 4 h after mitogen addition. However, while CGF was mitogenic for gingival fibroblasts, TGF-β was not. Kinetics of DNA stimulation and experiments with anti-PDGF antibodies indicated that PDGF-A expression does not contribute significantly to CGF-induced DNA synthesis. When the stimulation of various signaling pathways induced by CGF and other growth factors was compared, the pattern of stimulation by CGF was different from other growth factors. The characteristic signaling reactions of CGF are likely to be important components of the mechanisms that regulate the formation and regeneration of cementum and adjacent connective tissues.

A variety of polypeptide growth factors and cytokines regulate the migration, attachment, and growth of fibroblasts and their matrix synthesis. Many of these substances, which include PDGF, TGF-α, TGF-β, IL-1, and interferons, are secreted by platelets, macrophages, and other inflammatory cells during inflammation, and they are believed to influence the events involved in wound healing and tissue repair (1). Other growth factors, especially acidic and basic fibroblast growth factors and TGF-β are sequestered in the extracellular matrix, where the matrix appears to store and protect these molecules from denaturation and degradation (2–5).

Activation of cells to initiate DNA synthesis and complete cell division is associated with a series of signaling events that include PIP₃ hydrolysis and generation of IP₃ and 1,2-diacylglycerol, mobilization of Ca²⁺, PKC activation, and transcription of c-myc, c-fos, jun, and other protooncogenes (6, 7). These reactions are initiated by the binding of growth factors to their specific receptors. So far, two major signal transduction mechanisms have been shown to be associated with initiation of DNA synthesis and cell division. The first is autophosphorylation of some growth factor receptors at specific tyrosyl residues; the receptors then undergo dimerization and ligand-dependent association with SH-2 domains of PI-kinase, GTPase-activating protein, and PI-specific phospholipase Cγ-1 and activate these enzymes (8–11). This appears to be the major pathway of action of a group of mitogens, which includes PDGF, EGF, and IGF-1. The receptors for these proteins contain an inherent growth factor-dependent protein tyrosine kinase activity (8). In contrast, substances such as bombesin, thrombin, and bradykinin catalyze the breakdown of PIP₃ by a mechanism independent of receptor phosphorylation. This occurs through a family of GTP-binding proteins, some of which are inactivated by pertussis and cholera toxins (12–14). Irrespective of apparent triggering of a major signaling pathway, most growth factors activate a multitude of reactions, and no single event appears to be sufficient or necessary for the completion of DNA synthesis and cell division (15–23). Many of the signaling reactions are also redundant, and they are affected by the type and concentration of growth factors, duration of exposure to them, and the target cell type.

Cementum is the junctional interface through which collagen fibers of adjacent soft connective tissues are inserted into tooth root surfaces. It is a unique mineralized tissue which contains very few cells and is devoid of blood supply. The structural integrity of cementum is essential for the mobility and function of teeth and pathological alterations of cementum are...
human gingival fibroblasts were measured with the fluorescent Ca\(^{2+}\) indicator Indo-1. Subconfluent fibroblasts on glass coverslips (Nunc) were incubated in serum-free medium for 24 h, and the medium was replaced with Hanks' balanced salt solution containing 1 mg/ml BSA. The cells were exposed to 2 \(\mu\)M Indo-1-AM for 1 h at 37 °C, then rinsed with Hanks' balanced salt solution containing 1 mg/ml BSA, and maintained at 37 °C. Single cells were monitored for their degree of stimulation are distinct from other known growth factors, and that it may be a novel mitogen present in cementum but not in adjacent structures (28). Because CGF is the major growth factor present in cementum (26), we have performed experiments to understand its mode of action. Our results show that this growth factor resembles other mitogens in inducing a battery of signaling events, yet the type of reactions catalyzed and their degree of stimulation are distinct from other known growth factors.

**EXPERIMENTAL PROCEDURES**

**Materials—**c-fos and jun-B cDNAs were generous gifts from Dr. David Morris, Department of Biochemistry, University of Washington. Indo-1-AM was purchased from Molecular Probes, Junction City, OR. Peptides, Arg-Arg-Leu-Ser-Leu-Arg-Ala-Thr-Lys-Asp-Ala-Ala (5A peptide), Pro-Leu-Ala-Arg-Thr-Leu-Val-Ala-Gly-Leu-Pro-Gly-Lys-lys (sytide-2), and Arg-Arg-Arg-Glu-Thr-Arg-Arg-Arg-Glu-Arg-Ala-Ser-Thr-Ser-Lys-Pro-Leu-Ala-Arg-Thr-Leu-Ser-Lys-Asp-Ala-Ala (5A peptide) were synthesized in Howard Hughes Medical Institute, University of Washington. Anti-phosphotyrosine antibody was a gift from Dr. P. Rabinovich, Department of Pathology, and \(^{32}P\)J-protein A was purchased from ICN, Irvine, CA. \(^{131}I\)J-protein A was purchased from Amersham Corp. TPA, bombesin, and H\(_2\) were from Sigma. All other chemicals were of analytical grade.

**Purification of CGF—**CGF was obtained from human cementum as described previously (27). Briefly tissue was harvested, extracted in 1.0 M CH\(_3\)COOH containing a battery of proteinase inhibitors, fractionated by heparin-affinity HPLC, and subjected to precipitation by trichloroacetic acid and cation exchange and Cl\(_2\) reverse-phase HPLC. For experiments described here we used pooled C\(_3\) HPLC fractions that contained CGF and an additional M, 14,000 component, which was not mitogenic (27).

**Assay of DNA Synthesis—**Human gingival fibroblasts between 4th and 12th transfers were used as target cells. Assays were performed in quadruplicate in 96-well dishes (Falcon) using 10° cells/well. The cells were made quiescent by incubation in serum-free Dulbecco's modified Eagle's medium for 24-48 h and then mitogens added. After 22 h fresh serum-free medium with 10 \(\mu\)Ci/ml \(^{3}H\)thymidine was added. Six h later the cells were washed with ice-cold 0.1 M NaH\(_{2}\)PO\(_{4}\), pH 7.2, buffer containing 0.15 M NaCl and 5% trichloroacetic acid, taken in 1.0 N NaOH, neutralized, and counted in a Packard 1500 Tri-Carb liquid scintillation counter (26, 27).

**Measurement of Intracellular Calcium—**Intracellular Ca\(^{2+}\) levels of...
because, although the CGF is mitogenic by itself, it manifests
mitogenic activity as much as serum, caused only a slight
increase over that obtained with 10% serum controls (26-28):volatile to particular events, we compared cells exposed to CGF
obtained with EGF alone (Fig. 1A). Membrane activity levels were greater in the presence of CGF than with EGF (Fig. 2B); this increased only slightly when both CGF and EGF were used.

A common growth factor response is activation of serine/threonine protein kinases; therefore, we measured cytosolic seine/threonine kinase activities using myelin basic protein
(MAP-2 kinase), S6 kinase peptide, syntide-2, and casein kinase II peptide as substrates. Activities of the kinases for these substrates were increased 10 min after adding CGF or EGF. The levels in the presence of EGF, CGF, and CGF and EGF were not statistically different for the three different combinations (Fig. 3). CGF did not activate casein kinase II (Fig. 3D).

Expression of c-fos and jun family of cellular protooncogenes is associated with early mitogenic signaling, and the protein products of these genes form the AP-1 transcriptional-activating factor complex (7, 38, 39). To examine whether CGF activates transcription of these genes, Northern blots were performed using c-fos and jun-B cDNAs as representative probes in cells exposed to mitogens. No c-fos mRNA was detectable in quiescent gingival fibroblasts (Fig. 4, a–c). It was detectable after 30-min exposure to CGF, reached peak

FIG. 1. Effect of CGF, EGF, and CGF and EGF (A) and 10% fetal bovine serum (B) on cytosolic free Ca**+ levels in human gingival fibroblasts. Cells were grown on individual glass coverslips and loaded with Indo-1 as described under "Experimental Procedures." Individual cells were monitored for changes in Indo-1 fluorescence by an interactive laser cytometer ACAS570. The data represent a typical profile obtained for three independent experiments. CGF and EGF were added at 10 and 5.0 ng/ml, respectively, at times indicated by the arrows. FBS, fetal bovine serum.

RESULTS

We first examined whether CGF activates early signaling events that are associated with mitogenic stimulation. To determine whether changes in DNA synthesis can be attributed to particular events, we compared cells exposed to CGF alone and CGF plus EGF. The latter treatment was included because, although the CGF is mitogenic by itself, it manifests synergism with EGF, and its activity is potentiated to levels greater than that obtained with 10% serum controls (26-28).4

The response of other growth factors were also used as controls when necessary. CGF caused a rapid increase in cytosolic Ca**+ concentration, which reached 180% of initial value 80 s after mitogen addition and returned to basal level in the next 200 s (Fig. 1A). In contrast, 10% fetal bovine serum caused a 5-fold increase in Ca**+ level (Fig. 1B). However, CGF plus EGF treatment, which stimulated DNA synthesis and had mitogenic activity as much as serum, caused only a slight increase over CGF. The latter was roughly equal to that obtained with EGF alone (Fig. 1A).

CGF also increased IP3, IP2, and IP1 levels; the increase began within 1 min and reached a maximum at 10 min (data not shown). This indicated PIP2 hydrolysis and generation of 1,2-diacylglycerol. The latter process, along with higher Ca**+ levels, activates PKC and causes redistribution of PKC from the cytosol to membranes (37); therefore, cytosolic and membrane PKC activities were measured. CGF induced mobilization of PKC activity from the cytosolic fraction to the mem-

4For three typical assays, stimulation of DNA synthesis ([3H]thymidine uptake) was 10.3 ± 4.0, 5.0 ± 1.9, and 38.0 ± 14.7-fold with 10 ng/ml CGF, EGF, and CGF and EGF, respectively; it was 49.7 ± 11.0-fold in the presence of 10% fetal bovine serum control.

FIG. 2. Time course of protein kinase C distribution in cytosol and membrane fractions. Human gingival fibroblasts were treated for indicated times with CGF (10 ng/ml), EGF (5 ng/ml), or CGF and EGF, cells were harvested, and cytosol and membrane PKC activities were measured as described under "Experimental Procedures." A, PKC activity in cytosol (O) and membrane (●) fractions obtained from cells activated by CGF. Similar patterns were obtained from EGF and CGF and EGF treatments. B, membrane PKC activity in cells treated with CGF (●), EGF (□), and CGF and EGF (○).

FIG. 3. Protein serine/threonine kinase activities of human gingival fibroblasts treated with BSA vehicle (N), 5 ng/ml EGF (E), 10 ng/ml CGF (C), or both (C+E). Data were corrected by subtracting values of phosphate incorporation measured in the absence of substrate. The data represent averages of three measurements for S6 peptide (A), syntide-2 (B), myelin basic protein (C), and casein kinase-II peptide (D). The results represent a typical experiment repeated thrice.
levels after 1 h, and became undetectable after 2 h (Fig. 4a).
Fetal bovine serum, PDGF, TGF-β, and IL-1 also increased c-fos mRNA, although in the presence of these substances peak levels were reached earlier at 30 min (data not shown). By densitometry the increase in c-fos mRNA levels was calculated to be 10-, 9-, 19-, and 20-fold, respectively, for CGF, EGF, CGF and EGF, and serum treatments. The expression of jun-B mRNA, another early response gene, was also transiently induced although in this case maximum levels were present 2 h after mitogen addition (Fig. 4, d-f). The mRNA level of glucose-6-phosphate dehydrogenase demonstrated no differences for any of the conditions, confirming an equal load of all lanes.

These data showed that CGF stimulated early events associated with mitogenic stimulation. Growth factors such as PDGF, EGF, colony-stimulating factor, and insulin-like growth factor-I mediate these events through receptor tyrosine autophosphorylation, resulting in the stimulation of respective protein tyrosine kinase activity, which in turn tyrosine phosphorylates several cytoplasmic proteins (7–11). To examine whether CGF action is also mediated through receptor tyrosine phosphorylation, fibroblasts were exposed to CGF and tyrosine phosphorylation determined by Western blotting as described under “Experimental Procedures.” PDGF6 and EGF treatments were compared as controls, because products of these mitogens have been identified. Two protein bands at \( M, 170,000-180,000 \) and \( \sim 300,000 \) (Fig. 5, bands \( b \) and \( a \), respectively) were prominent in cells exposed to PDGF, and in addition several other protein bands were also present migrating with \( M, 140,000, 85,000, 77,000, 60,000, 52,000, 49,000, \) and \( 42,000 \) (Fig. 5, bands \( c-i \), respectively). Similar bands were also present in the presence of EGF although \( d, e, \) and \( f \) migrated slightly differently (Fig. 5). In the presence of CGF the prominent bands of \( M, 180,000-300,000 \) (bands \( a \) and \( b \)) were not detectable. All others were present although the degree of stimulation of bands \( f-i \) was considerably less and only barely detectable. Cells treated with both CGF and EGF had a pattern identical to that obtained for EGF and no additional bands were detected (data not shown).

The absence of any novel tyrosine-phosphorylated protein bands in cells exposed to CGF indicated that receptor tyrosine phosphorylation probably does not participate CGF-induced signaling reactions to a significant extent. Polypeptides such as bombesin and thrombin catalyze PI hydrolysis and subsequent signaling events through a receptor-tyrosine kinase independent mechanism, which is sensitive to pertussis toxin (12–14). Therefore we examined whether CGF-mediated DNA synthesis is affected by pertussis toxin. Quiescent gingival fibroblasts were treated with 5 ng/ml pertussis toxin as described in Table I, and DNA-synthesis was measured. The results showed that this toxin reduced DNA synthesis by 65% (Table I). The inhibition was 61% for serum. Bombesin had very little mitogenic activity at concentrations from 1 to 100 nM tested, alone, or with EGF, and the small thymidine

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**Fig. 4. Northern analysis of c-fos and jun-B mRNA levels in human gingival fibroblasts at the indicated times following treatment with CGF, EGF, and CGF+EGF.** In each lane, 15 μg of total RNA was fractionated on a 1% agarose gel and transferred onto a Nitran filter. The filter was hybridized with \(^3^2^P\)-labeled c-fos and jun-B probes and exposed at −70 °C for 36 and 48 h, respectively.

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**Fig. 5. Tyrosine phosphorylation of cellular proteins in human gingival fibroblasts treated with EGF, PDGF, or CGF.** Cells were treated with 5, 5, and 10 ng/ml mitogens, respectively, for 5, 10, and 20 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting using anti-phosphotyrosine antibody. Bands were visualized with \(^1^2^5^I\)-Protein A as described under “Experimental Procedures.” The migration of molecular mass markers is indicated on the left. The bands \( a-i \) represent \( M, 300,000, 180,000, 140,000, 85,000, 77,000, 60,000, 52,000, 49,000, \) and \( 42,000 \), respectively.

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**Table I**

| Addition       | \[^3^H\]Thymidine uptake* |
|----------------|--------------------------|
|                | −            | +       | %    |
| CGF            | 1.0 ± 0.1 | 0.3 ± 0.1 | 35 ± 5 |
| EGF            | 2.0 ± 0.1 | 1.4 ± 0.1 | 69 ± 9 |
| CGF and EGF   | 13.1 ± 0.5 | 8.8 ± 0.2 | 67 ± 12 |
| PDGF           | 1.0 ± 0.1 | 0.6 ± 0.1 | 62 ± 7 |
| FBS\(^6\)      | 6.4 ± 0.1 | 1.6 ± 0.2 | 39 ± 12 |
| Bombesin\(^5\) | 0.1 ± 0.1 | 0.03 ± 0.04 | 30 ± 40 |

*Quiescent human gingival fibroblasts were first treated with 5 ng/ml pertussis toxin for 1 h in serum-free medium containing 1.0 mg/ml BSA. After the pretreatment mitogens were added and \[^3^H\] thymidine uptake determined as described under “Experimental Procedures.” Numbers represent mean ± S.D. of triplicates and those under and + are cpm ± S.D. \( \times 10^{-4} \) of treatments without and with pertussis toxin, respectively. Representative data from one experiment are given.

\(^5\) 10% fetal bovine serum.

\(^6\) 100 nM.
PKC activation occupies a central role in mediating several cell proliferation reactions (40, 41). However, in some cells these events can be mediated through a PKC-independent pathway, and growth factors could be distinguished based on the susceptibility of their action to PKC inhibitors (14, 19, 42). To examine whether CGF response is related to PKC activation, before mitogen addition fibroblasts were either exposed for 2 h to 50 μM H7, a PKC inhibitor, or treated with 162 nM TPA (100 ng/ml) for 24 h to down-regulate PKC (43). Measurement of DNA synthesis showed that it was 14–17, 35–50, and 52–68% as much as untreated controls for the EGF, PDGF, and fetal bovine serum treatments, respectively (Table II). In contrast, in cells exposed to CGF 71–75% of the DNA synthesis remained after PKC inhibition.

To determine if an increase in any of the various activities measured correlated directly with the stimulation in DNA synthesis, the magnitudes of their increase was compared (Table III). The increase was 1–5-fold for Ca2+, IP3, serine-threonine kinases, and PKC levels in cells treated with CGF or EGF, and it was greater (up to 10-fold) for c-fos and jun-B mRNA. When both mitogens were present the increase was the sum obtained with each growth factor alone. No clear cut correlation was present between any of the activity measured and DNA synthesis. This was most notable in CGF and EGF treatments when the stimulation of DNA synthesis was considerably greater (Table III), indicating that other mechanisms may contribute to DNA synthesis. Induction of PDGF-A chain expression is one such mechanism that is believed to contribute to mitogenic activity of IL-1, PDGF-AB, and TGF-β (44–46). We therefore performed experiments to determine the levels of PDGF-A mRNA expression in cells treated with CGF. TGF-β treatment was included as a control as this growth factor activates PDGF-A mRNA expression, but it is not mitogenic for gingival fibroblasts (45–47). As expected, TGF-β caused a significant increase in PDGF-A mRNA level, which reached maximal levels at 2–4 h after its addition (Fig. 6). CGF also caused an increase at 4 h, although it was less. In contrast, EGF had no significant effect either alone or in the presence of CGF (Fig. 6). The increase in PDGF-A chain mRNA levels caused by TGF-β, CGF, and CGF and EGF was 14–5-, and 5-fold, respectively. PDGF-AB, serum, and IL-1/β also enhanced PDGF-A mRNA levels (data not shown). None of these growth factors had any effect on PDGF-B mRNA levels (data not shown). The levels of PDGF in the cells and secreted into the media were also determined in cells treated with different growth factors (Table IV). Although increased levels of PDGF in the media were observed in all cases where PDGF-A chain mRNA expression was induced, the relative increase in PDGF mRNA levels did not correlate with the amount of PDGF detected in the media or cells.

To determine whether the appearance of PDGF-A chain and its release into media leads to a second wave of mitogenic activity, DNA synthesis kinetics were assessed. However, no evidence for secondary induction was observed as DNA synthesis occurred at 24 h for all mitogenic stimulants (Fig. 7). TGF-β, as expected, had no significant mitogenic activity.

**DISCUSSION**

Our data show that CGF induces many of the signaling pathways associated with mitogenesis. These pathways include an increase in cytosolic Ca2+ concentration, PI hydrolysis, activation of PKC cascade, and expression of cellular protooncogenes. Several tyrosine-phosphorylated proteins were also formed. While these data show that CGF resembles other growth factors in activating early signaling pathways, there are distinct differences. For example, we were unable to detect any new tyrosine-phosphorylated protein that may represent putative CGF receptor. Although low level receptor expression may prevent detection of the phosphorylated receptor, the data indicate that CGF is different from PDGF-EGF group of growth factors in mediating signaling events through a mechanism independent of receptor tyrosine phosphorylation. The absence of receptor tyrosine phosphorylation may be a reason why tyrosine phosphorylation of bands e–i (Fig. 5) is considerably less than that observed with EGF and PDGF.

CGF also appears to differ from thrombin-bombesin-like mitogens. For example, bombesin has very little mitogenic activity toward human gingival fibroblasts. While bombesin-induced mitogenic activity is completely susceptible to pertussis toxin in Swiss 3T3 cells (14), 35% of DNA synthesis induced by CGF is not sensitive to pertussis toxin. Thus, CGF signaling appears to involve both pertussis toxin-sensitive guanidine nucleotide-binding proteins as well as pertussis toxin-independent pathways. Although CGF, EGF (Figs. 2 and 3), PDGF, and serum treatments (data not shown) activate protein kinases to a similar extent, the inhibition by PKC inhibitors for CGF is less than for other growth factors (Table II) (14). This appears contradictory. One possible reason for this may be the ability of CGF to trigger multiple signaling events that complement and substitute the action of protein kinases. While susceptibility to inhibitors may depend on many factors such as target cell type, effector concentration, duration of exposure, etc., the overall pattern of susceptibility of CGF response to these inhibitors is characteristic and different from other growth factors and provides

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**Table II**

| Addition | **[H]Thymidine uptake** |
|----------|-------------------------|
|          | [H]H7 | [H]H7 + TPA* |
| CGF      | 0.7 ± 0.1 | 0.5 ± 0.1 |
| EGF      | 1.9 ± 0.1 | 0.6 ± 0.1 |
| CGF + EGF| 11.7 ± 0.2 | 4.0 ± 0.1 |
| PDGF     | 0.9 ± 0.1 | 0.4 ± 0.1 |
| FBS†     | 5.5 ± 0.5 | 4.2 ± 0.2 |

*After TPA treatment PKC activity of human gingival fibroblasts (cytosol plus membrane fractions) was 17 ± 15% (n = 3) as much untreated cells.

†Cells were pretreated with 50 μM H7 or 100 ng/ml (180 nM) TPA for 2 and 24 h, respectively, before measuring DNA synthesis. The numbers in + and columns are cpm ± S.D. of triplicates from one of three representative experiments without or with treatments, respectively. The percent values are mean ± S.D. of three separate experiments each consisting of triplicates.
TABLE III

Stimulation of signaling pathways and DNA synthesis in human gingival fibroblasts by CGF and other mitogens

Representative data from one experiment are given.

| Growth factor | Fold stimulation by |
|---------------|---------------------|
| CGF | EGF | CGF and EGF | 10% FBS |
| Ca²⁺ | 1.6 | 1.2 | 1.8 | 3.8 |
| IP | 3.2 | 2.2 | 5.1 | ND⁺ |
| S₃ kinase | 1.9 | 2.1 | 2.2 | ND⁺ |
| PKC | 3.9 | 3.2 | 4.3 | ND⁺ |
| c-fos mRNA | 10.2 | 9.0 | 18.5 | 24.3 |
| jun-B mRNA | 7.1 | 2.2 | 8.9 | 7.7 |
| DNA synthesis | 8.1 | 3.8 | 41.4 | 36.8 |

* ND, not done.
⁺ Measured by densitometry.

![Northern analysis for PDGF-A chain mRNA levels in human gingival fibroblasts at the indicated times following treatment with CGF, TGF-β, CGF and EGF, or EGF. 15 µg of total RNA was fractionated in each lane on a 1% agarose gel and transferred onto a Nytran filter. The filter was hybridized with [32P] PDGF-A chain probe and exposed at -70 °C for 36 h.](image1)

![Time course of [³H]thymidine incorporation by human gingival fibroblasts treated with CGF (●), CGF and EGF (○), TGF-β (△), or no mitogens (con) (□).](image2)

**Fig. 6. Northern analysis for PDGF-A chain mRNA levels in human gingival fibroblasts treated with CGF, TGF-β, CGF and EGF, or EGF.**

**Fig. 7. Time course of [³H]thymidine incorporation by human gingival fibroblasts treated with CGF (●), CGF and EGF (○), TGF-β (△), or no mitogens (con) (□).**

additional support for the possibility that CGF may be a novel growth factor.²

The stimulation of various activities measured in the presence of both CGF and EGF is roughly equal to that obtained with each growth factor alone, while DNA synthesis is enhanced to a considerably greater extent (Table III). Tyrosine phosphorylation of several proteins in the presence of CGF and EGF was comparable with EGF, yet the former treatment was considerably more mitogenic. The increase in c-fos and jun-B levels also do not appear to match DNA synthesis, especially with TGF-β and IL-1 (data not shown), which induce these genes but not DNA synthesis. These results are consistent with other studies that show that many of the individual signaling events are not rate limiting for DNA synthesis and that DNA synthesis does not depend upon a single pathway (15–22, 43, 48–50). For example, other mechanisms, phosphatidylcholine hydrolysis and arachidonic acid formation for example (51), may also contribute to DNA synthesis.

Although CGF induces the expression of PDGF-A chains, we do not believe that this process contributes significantly to DNA synthesis. This is because (i) TGF-β and IL-1, which are more potent in inducing the PDGF-A mRNA levels than CGF, have very little mitogenic activity for gingival fibroblasts (Fig. 7) (47); (ii) PDGF-A at 5× concentration detected in conditioned media had negligible mitogenic activity (data not shown); (iii) no significant peak of DNA synthesis was observed at times corresponding to the induction of the PDGF-A mRNA (Fig. 7); (iv) antibodies that recognize PDGF-A do not significantly inhibit thymidine uptake induced by the CGF (27). Interestingly, while EGF increases the expression of PDGF-A mRNA in other cells (45), it has no effect on human gingival fibroblasts.

CGF is the major mitogen present in cementum matrix, and it is not detected in adjacent dentin or soft tissues (28). Its unique localization raises several possibilities about its function. For example, it may be stored in the matrix in a manner similar to FGFs, released at a time of need (during inflammation, for example), and it may participate in the formation and regeneration of soft periodontal connective tissues. Its ability to induce the expression of PDGF-A, a mitogen that appears early in inflammation (52) and its stimulation of collagen synthesis better than BB or AB dimers in human lung fibroblasts,⁷ may be significant in this regard. Additionally, the CGF may promote the migration and growth of progenitor cells present in adjacent structures toward the dentin matrix and participate in their differentiation into cementoblasts (53). The variety of signaling mechanisms induced by CGF may contribute to a pleiotropic influence, and

⁷ G. Raghu and A. S. Narayanan, unpublished data.
in these actions it may be aided by other growth factors that are also present in the cementum matrix (26).

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