Brain-derived Neurotrophic Factor Stimulates Bone/Cementum-related Protein Gene Expression in Cementoblasts*

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Brain-derived neurotrophic factor (BDNF), recognized as essential in the developing nervous system, is involved in differentiation and proliferation in non-neuronal cells, such as endothelial cells, osteoblasts, and periodontal ligament cells. We have focused on the application of BDNF to the regeneration of periodontal tissue and indicated that BDNF promotes the regeneration of experimentally created periodontal defects. Cementoblasts form cementum, mineralized tissue, which is key to establishing a functional periodontium. The application of BDNF to the regeneration of periodontal tissue requires elucidation of the mechanism by which BDNF regulates the functions of cementoblasts. In this study, we examined how BDNF regulates the mRNA expression of bone/cementum-related proteins (alkaline phosphatase (ALP), osteopontin (OPN), and bone morphogenetic protein-2 (BMP-2)) in cultures of immortalized human cementoblast-like (HCEM) cells. BDNF elevated the mRNA levels of ALP, OPN, and BMP-2 in HCEM cells. Small interfering RNA (siRNA) for TRKB, a high affinity receptor of BDNF, siRNA for ELK-1, which is a downstream target of ERK1/2, and PD98059, an ERK inhibitor, obviated the increase in the mRNA levels. BDNF increased the levels of phosphorylated ERK1/2 and Elk-1, and the blocking of BDNF signaling by treatment with siRNA for TRKB and PD98059 suppressed the phosphorylation of ERK1/2 and Elk-1. Furthermore, BDNF increased the levels of phosphorylated c-Raf, which activates the ERK signaling pathway. These findings provide the first evidence that the TrkB-c-Raf-ERK1/2-Elk-1 signaling pathway is required for the BDNF-induced mRNA expression of ALP, OPN, and BMP-2 in HCEM cells.

Brain-derived neurotrophic factor (BDNF),2 cloned in 1989 as the second member of the neurotrophin family, plays a role in the survival and differentiation of central and peripheral neurons through binding to a product of TrkB, a high affinity receptor (1–4). BDNF also binds to p75NTR, a low affinity receptor (5, 6). Various types of non-neural cells and tissue express BDNF. At the cellular level, vascular endothelial cells, osteoblastic cells, periodontal ligament cells, and immune cells express BDNF (7–11). At the tissue level, BDNF is expressed in tooth germ, bone, cartilage, heart, spleen, placenta, prostate, and kidney (12–16).

Cementum, produced by cementoblasts, is a mineralized tissue that forms the outer covering of anatomic tooth roots. Its formation is key to establishing functional periodontal tissue, which is structured with newly formed cementum, alveolar bone, and connective tissue fibers inserted into these hard tissues. We have focused on using BDNF to help regenerate periodontal tissue and found that the regulation of functions of periodontal ligament cells and endothelial cells by BDNF culminates in the promotion of periodontal tissue regeneration in vitro and in vivo (10). Furthermore, cementum forms in the early stages of the regeneration stimulated by BDNF (10). The mechanism whereby BDNF regulates cementoblast functions should be elucidated.

Human cementoblast-like (HCEM) cells have been established by transfection of hTERT (17). The cells express bone/cementum-related proteins, such as type I collagen, runt-related transcription factor 2, osteocalcin, bone sialoprotein, and CP-23, show strong ALP activity and form calcified nodules.

The extracellular signal-regulated kinase (ERK) pathway plays an important role in the differentiation of postmitotic cells. For example, in Xenopus laevis embryos, inhibition of the activation of ERK1/2 prevents animal caps from differentiating into mesenchymal tissue (18), and mice harboring deletions in ERK2 exhibit severe defects in primary mesenchyme formation without major changes in cell proliferation or apoptosis (19). In osteoblasts, the ERK1/2 pathway is a major conduit for conveying information about the extracellular environment to the nucleus. Osteoblasts respond to various stimuli, such as hormone/growth factors, extracellular matrix-integrin binding, androgens; MEM, minimum Eagle’s medium; PBS, phosphate-buffered saline; PDTC, pyrrolidinedithiocarbamate; BMP, bone morphogenetic protein; MAPK, mitogen-activated protein kinase; FGF, fibroblast growth factor; ERK, extracellular signal-regulated kinase.

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2 The abbreviations used are: BDNF, brain-derived neurotrophic factor; OPN, osteopontin; ALP, alkaline phosphatase; HCEM, human cementoblast-like; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, minimum Eagle’s medium; PBS, phosphate-buffered saline; PDTC, pyrrolidinedithiocarbamate; BMP, bone morphogenetic protein; MAPK, mitogen-activated protein kinase; FGF, fibroblast growth factor; ERK, extracellular signal-regulated kinase.

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and mechanical loading, and ERK1/2 is activated in the cells (20–25).

Elk-1 is a transcription factor and a downstream target of ERK1/2. c-Raf is a main effector recruited by GTP-bound Ras to activate the ERK1/2 signaling pathway. Elk-1 and c-Raf as well as ERK may be intercellular signaling molecules due to be stimulated by BDNF in cementoblasts.

In this study, to clarify the actions of BDNF in bone/cementum-related protein expression in cementoblasts, we investigate the mRNA expression of alkaline phosphatase (ALP), osteopontin (OPN), and bone morphogenetic protein-2 (BMP-2), and regulatory mechanisms in cultures of HCEM cells. For the first time, we demonstrate that BDNF promotes the mRNA expression of ALP, OPN, and BMP-2 in HCEM cells through a TrkB-c-Raf-ERK1/2-Elk-1 signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—HCEM cells, immortalized by transfection of the hTERT gene, were established by Kitagawa et al. (17). The cells were cultured in α-minimum essential medium (α-MEM, Sigma) supplemented with 10% fetal bovine serum (Invitro-Gen, Minneapolis, MN) for specified periods before the end of incubation in medium B. HCEM cells and SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium nutrient mixture Ham’s F-12, with 10% fetal bovine serum (Invitro-Gen, Minneapolis, MN) for specified periods before the end of incubation in medium B. Total RNA from each culture was extracted with ISOGEN® (Wako Pure Chemical, Osaka, Japan) and quantified by spectrophotometry at 260 and 280 nm.

Real Time PCR—mRNA expressions of ALP, OPN, and BMP-2 and mRNA expression knocked down by the siRNAs were quantified by real time PCR. The PCR was carried out in two steps with a Lightcycler system using SYBR Green (Roche Diagnostics). The sense primers and antisense primers used to detect the mRNA of ALP, BMP-2, OPN, TRKB, p75NTR, ELK-1, and GAPDH are listed in Table 1.

Immunoblotting of TrkB, p75NTR, Total Raf, and Phosphorylated c-Raf—HCEM cells with or without transfection of the siRNAs were seeded at a density of 1×10⁵ cells per well in 6-well plastic culture plates and maintained in medium A. The nontransfected cells or transfected cells were pretreated with or without PD98059 (50 μM) and PDTC (10 μM) for 30 min and then exposed to BDNF (20 ng/ml) for specific periods before the end of incubation in medium C. SH-SY5Y cells were seeded at a density of 1×10⁵ cells per well in 6-well plastic culture plates and maintained in medium B. Total RNA from each culture was extracted with ISOGEN® (Wako Pure Chemical, Osaka, Japan) and quantified by spectrophotometry at 260 and 280 nm.

The transfected cells were seeded at a density of 1×10⁵ cells per well in 6-well plastic culture plates and maintained in medium A. The HCEM cells were pretreated with or without PD98059 (50 μM, Calbiochem), SB203580 (10 μM, Calbiochem), SP600125 (10 μM, Calbiochem), or PDTC (10 μM, Calbiochem) for 30 min and then exposed to various concentrations of human recombinant BDNF (R & D Systems, Minneapolis, MN) for specified periods before the end of incubation in α-MEM supplemented with penicillin G solution (100 units/ml) and streptomycin (100 μg/ml) (medium C). SH-SY5Y cells were seeded at a density of 1×10⁵ cells per well in 6-well plastic culture plates and maintained for 7 days in medium B. Total RNA from each culture was extracted with ISOGEN® (Wako Pure Chemical, Osaka, Japan) and quantified by spectrophotometry at 260 and 280 nm.

Transfection of siRNAs into HCEM Cells—Electroporation with ECM 830® (BTX, Holliston, MA) was used for the transfection of each siRNA into HCEM cells. HCEM cells (1×10⁵) were suspended in 75 μl of siRNA Electroporation Buffer (Ambion) with 1.5 μg of each siRNA and placed into 1-mm cuvettes. Transfections were performed with 900 V, a 70-μs pulse length, and 2 pulses between 5 s. After the pulses, the cells were incubated for 8 min at 37 °C and suspended in medium A. The transfected cells were seeded at a density of 1×10⁵ cells per well in 6-well plastic culture plates and cultured in medium A. The effectiveness of the knockdown was determined by quantifying the level of the target genes and proteins by real time PCR and immunoblotting, respectively, as described below.

Isolation of Total RNA—HCEM cells with or without transfection of the siRNAs were seeded at a density of 1×10⁵ cells per well in 6-well plastic culture plates and maintained in medium A. The HCEM cells were pretreated with or without PD98059 (50 μM, Calbiochem), SB203580 (10 μM, Calbiochem), SP600125 (10 μM, Calbiochem), or PDTC (10 μM, Calbiochem) for 30 min and then exposed to various concentrations of human recombinant BDNF (R & D Systems, Minneapolis, MN) for specified periods before the end of incubation in α-MEM supplemented with penicillin G solution (100 units/ml) and streptomycin (100 μg/ml) (medium C). SH-SY5Y cells were seeded at a density of 1×10⁵ cells per well in 6-well plastic culture plates and maintained for 7 days in medium B. Total RNA from each culture was extracted with ISOGEN® (Wako Pure Chemical, Osaka, Japan) and quantified by spectrophotometry at 260 and 280 nm.

The samples were then microcentrifuged at 13,000 rpm for 10 min at 4 °C and heated at 95 °C for 5 min. Samples were resolved on a 12% SDS-PAGE under nonreducing conditions and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% skim milk for 1 h and then reacted with mouse anti-human TrkB antibody (R & D Systems; 1:250), rabbit anti-human p75NTR antibody (Abcam, Cambridge, MA; 1:1000), rabbit anti-human phosphorylated c-Raf Ser-338 antibody (Cell Signaling, Beverly, MA; 1:1000), rabbit anti-human total c-Raf antibody (Cell Signaling, 1:1000); and mouse anti-human β-actin antibody (Zymed Laboratories Inc.; 1:2000). After the mem-

| Target gene | Primer sequence |
|-------------|-----------------|
| ALP         | Forward, 5′-GCGCCGAACAAAGGAAATG-3′ |
| BMP-2       | Forward, 5′-GCATGTCGCCGCTTGGATG-3′ |
| OPN         | Forward, 5′-GATGGCGAGAGTAGATGTTG-3′ |
| TRKB        | Forward, 5′-CTGTCGCGGAAAAATCTTTGA-3′ |
| p75NTR      | Reverse, 5′-ACCCGTATTAGACCACTGAA-3′ |
| ELK-1       | Reverse, 5′-ACCCGTATTAGACCACTGAA-3′ |
| GAPDH       | Forward, 5′-AAGGTTCGGAAGGCACCCTG-3′ |
|             | Reverse, 5′-AGTTGCTTCTGCTTGGTACG-3′ |

| TABLE 1 | Sense primers and antisense primers for real time PCR |
|---------|-----------------------------------------------------|
| Target gene | Primer sequence |
| ALP      | Forward, 5′-GCGCCGAACAAAGGAAATG-3′ |
| BMP-2    | Forward, 5′-GCATGTCGCCGCTTGGATG-3′ |
| OPN      | Forward, 5′-GATGGCGAGAGTAGATGTTG-3′ |
| TRKB     | Forward, 5′-CTGTCGCGGAAAAATCTTTGA-3′ |
| p75NTR   | Reverse, 5′-ACCCGTATTAGACCACTGAA-3′ |
| ELK-1    | Reverse, 5′-ACCCGTATTAGACCACTGAA-3′ |
| GAPDH    | Forward, 5′-AAGGTTCGGAAGGCACCCTG-3′ |
|          | Reverse, 5′-AGTTGCTTCTGCTTGGTACG-3′ |
brane was washed, it was incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Biosciences) or horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling) in Tris-buffered saline (TBS; 20 mM Tris-HCl, 0.15 M NaCl (pH 7.6)) for 1 h at room temperature. After further washing, immunodetection was performed by using ECL Plus Western blotting detection reagents (Amersham Biosciences) or a chemiluminescence system (Cell Signaling).

Calcification Assay—Calcification was detected by Dahl’s method for calcium (26). HCEM cells were placed in a 24-well plate at a density of 1 × 10⁵ cells/well and cultured in α-MEM supplemented with 5% fetal bovine serum, 50 µg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, and 10 mM dexamethasone (mineralizing media), including BDNF (20 ng/ml), PD98059 (50 µM), or PDTC (10 µM) at 37 °C. Media were changed every other day, and the cells were maintained for 14 days. The cells were fixed in 3.7% formaldehyde neutral buffer solution and then stained with alizarin red S.

Preparation of Cytoplasmic and Nuclear Extracts—Cytoplasmic and nuclear protein extracts were fractionated using NE-PER® extraction reagents (Pierce). The HCEM cells were scraped off the dish and collected by centrifugation at 500 × g for 3 min. Subsequent steps for fractionation were performed according to the manufacturer’s instructions. Protein concentration was determined using Bio-Rad Protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

ERK1/2 Activity and Total ERK1/2 Expression—To examine ERK1/2 activity, a p44/42 MAPK assay kit (Cell Signaling) was used. HCEM cells were collected with 500 µl of cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 µM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and sonicated four times for 5 s each time on ice. The samples were then microcentrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant was transferred to a new tube. For the assay of ERK activity, the supernatant was immunoprecipitated with anti-p44/42 (Thr-202/Thr-204) monoclonal antibody. After an overnight incubation, beads were microcentrifuged for 30 s at 4 °C. The collected beads were washed four times and suspended in 50 µl of kinase buffer supplemented with 200 µM ATP and 2 µg of Elk-1 fusion protein for p44/42, before being incubated for 30 min at 30 °C. The reaction was terminated by adding 3× SDS sample buffer. From each sample, 30 µl was then loaded on a 10% SDS-polyacrylamide gel. After transfer, membranes were blocked for 3 h at room temperature in TBS/Tween (0.05%) containing 5% skim milk. Membranes were then incubated with primary antibody (rabbit anti-phospho-Elk-1 antibody; 1:1000) diluted in primary antibody buffer (TBS-T, 5% bovine serum albumin) overnight at 4 °C. Membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000) in blocking buffer. After further wash-
Action of BDNF on Human Cementoblast-like Cells

FIGURE 2. BDNF enhances bone/cementum-related protein mRNA expression in HCEM cells via TrkB.

A, time course effect. HCEM cells were exposed to BDNF (20 ng/ml) for the periods indicated before the end of incubation on day 7. B, dose-dependent effect. HCEM cells were exposed to increasing concentrations of BDNF for 12 h before the end of incubation on day 7. Graphs show the ratio of OPN, ALP, or BMP-2 mRNA to GAPDH mRNA. Values represent means ± S.D. of three cultures. *, p < 0.01; value differs significantly from the control (analysis of variance). C and E, effectiveness of siRNA of TRKB and p75NTR. HCEM cells, having been transfected with negative control (Neg), TRKB, or p75NTR siRNA, were cultured for 2 days. The effectiveness of the siRNAs was confirmed by real time PCR. Graphs show the ratio of TRKB or p75NTR mRNA to GAPDH mRNA. Values represent means ± S.D. of three cultures. *, p < 0.01; value differs significantly from the control (t test).

BMP-2 expression was determined by analysis of variance or Student’s t test.

RESULTS

Expression of TrkB and p75NTR Receptors in HCEM Cells—Real time PCR indicated that HCEM cells as well as SH-SY5Y cells for a positive control expressed mRNA of TRKB, and p75NTR and p75NTR mRNA levels were higher than TRKB mRNA levels in both cells (Fig. 1A). In addition, the ratio of the TRKB mRNA levels to p75NTR mRNA levels in HCEM cells was similar to that of SH-SY5Y cells (Fig. 1A). Furthermore, immunoblotting showed protein expression of TrkB and p75NTR in HCEM cells and SH-SY5Y cells (Fig. 1B). Regarding the expression of TrkB, the typical three-band pattern, three forms of 80, 110, and 140 kDa were detected in both cells. Thus, the
expression pattern of TrkB and p75NTR in HCEM cells was similar to that in SH-SY5Y cells. The protein expression of TrkB and p75NTR in HCEM cells was confirmed by immunofluorescence microscopy (Fig. 1, C, left panel, and D, left panel). In addition, TrkB partially co-localized with p75NTR (Fig. 1E). In contrast, no proteins were detected by immunofluorescence with anti-nonimmune isotype-matching IgG (Fig. 1, C, right panel, and D, right panel).

TrkB Is Involved in the BDNF-induced Increase in Bone/Cementum-related Protein mRNA Levels in HCEM Cells—The time course experiments showed that BDNF at 20 ng/ml increased mRNA levels of OPN, ALP, and BMP-2 in a time-dependent manner until 12 h (Fig. 2A). The dose-experiment showed that BDNF at 20 ng/ml caused a 2.2-fold increase in OPN mRNA expression, a 2.9-fold increase in ALP mRNA expression, and a 3.2-fold increase in BMP-2 mRNA expression with a maximal effect (Fig. 2B). To examine which receptor (that of TrkB or p75NTR) is most involved in the BDNF-induced enhancement of bone/cementum-related protein mRNA expression, siRNA of TRKB or p75NTR was transfected into HCEM cells. Two siRNAs for knockdown of TRKB and p75NTR were used. The mRNA expression of TRKB and p75NTR in HCEM cells was markedly down-regulated by transfection of TRKB siRNA and p75NTR siRNA, respectively (Fig. 2, C and E). On the other hand, TRKB siRNA treatment and p75NTR siRNA treatment did not influence mRNA expression of p75NTR (Fig. 2C) and TRKB (Fig. 2E), respectively. Furthermore, depletion of TrkB and p75NTR protein expression in HCEM cells following the treatment of the siRNAs was confirmed by immunoblotting (data not shown). The knockdown of TRKB, but not p75NTR, abolished the BDNF-induced increase in bone/cementum-related protein mRNA levels (Fig. 2, D and F). Furthermore, the quite similar results were obtained with a second siRNA for TRKB or p75NTR.

Stimulation of Bone/Cementum-related Protein mRNA Expression by BDNF Depends on ERK1/2 Signaling—Our preliminary studies have shown that the ERK inhibitor (PD98059, 50 μM) does not impact cell viability of HCEM cells (data not shown). The ERK inhibitor abolished the increase in OPN, ALP, and BMP-2 mRNA levels induced by BDNF (Fig. 3A), whereas the NF-κB inhibitor (PDTC, 10 μM), the c-Jun NH2-terminal kinase (JNK) inhibitor (SP600125, 10 μM), and the p38 MAPK inhibitor (SB203580, 10 μM) did not affect the increase (Fig. 3B and data not shown). BDNF stimulated calcification identified by alizarin red S staining in cultures of HCEM cells. Furthermore, PD98059 but not PDTC suppressed calcification induced by BDNF (Fig. 3C). The effect of PD98059 on the BDNF-induced OPN, ALP, and BMP-2 mRNA expression was reversible. After PD98059 and BDNF were removed from the medium of HCEM cells having been exposed to them for 24 h, newly added BDNF increased mRNA levels of the cementum/bone-related proteins again
increase in the activity of phosphorylated ERK1/2 (Fig. 3, PDTC inhibited the activity of NF-
PD98059 and TRKB ERK1/2 in HCEM cells stimulated by BDNF (Fig. 3). In addition, transfection of TRKB siRNA resulted in the translocation of ERK1/2 to the nucleus by BDNF being inhibited (Fig. 4D). In contrast, transfection of the negative control siRNA did not influence the nuclear translocation of ERK1/2 (Fig. 4E). The results from the immunofluorescence microscopy were supported with immunoblotting of ERK1/2 in the nuclear fractions and cytoplasmic fractions in HCEM cells (Fig. 4F).

Elk-1, Downstream of the ERK1/2 Signaling Pathway, Is Involved in the BDNF-induced Increase in Bone/Cementum-related Protein mRNA Levels in HCEM Cells—The expression of phosphorylated Elk-1 was weak in unstimulated HCEM cells (Fig. 5A). In contrast, phosphorylated Elk-1 was clearly expressed in the nucleus of the cells treated with BDNF (Fig. 5B). Immunoblotting showed that the levels of phosphorylated Elk-1 in the nuclear fractions in HCEM cells in the presence of BDNF were higher than those in the absence of BDNF (data not shown). No expression of phosphorylated Elk-1 was detected in the presence of BDNF and PD98059 (Fig. 5C). Furthermore, the phosphorylated Elk-1 was not detected in the cells transfected with TRKB siRNA (Fig. 5D), but it was detected in the cells transfected with negative control siRNA (Fig. 5E). Expression of ELK-1 mRNA was down-regulated in the cells transfected with ELK-1 siRNA (Fig. 5F). The knockdown of ELK-1 completely abolished the BDNF-induced increase in OPN and ALP mRNA levels to the basal levels, whereas it partially abolished the increase in BMP-2 mRNA levels (Fig. 5G). Similar results were obtained from the experiment with a second siRNA for ELK-1 (data not shown).

Effect of BDNF on Expression of c-Raf, an Upstream Target of ERK1/2—BDNF at 20 ng/ml increased the level of c-Raf phosphorylated at Ser-338 in a time-dependent manner until 10 min (Fig. 6A). Transfection of TRKB siRNA resulted in inhibition of the phosphorylation of c-Raf in HCEM cells stimulated by BDNF (Fig. 6B). In contrast, p75NTR siRNA did not influence the phosphorylation of c-Raf in HCEM cells by BDNF stimulation (Fig. 6C). Similar results were obtained from the experiment with a second siRNA for TRKB or p75NTR (data not shown).

(data not shown). PD98059, PDTC, SP600125, and SB203580 did not influence the bone/cementum-related protein mRNA expression without BDNF (Fig. 3, A and B and data not shown).

Subsequently, we examined ERK1/2 activity by immunoblotting. BDNF at 20 ng/ml increased the activity of phosphorylated ERK1/2, and the maximal effect was seen at 10 min (Fig. 3D). PD98059 and TRKB siRNA abrogated the BDNF-induced increase in the activity of phosphorylated ERK1/2 (Fig. 3, E and F). PDTC did not influence the activity of phosphorylated ERK1/2 in HCEM cells stimulated by BDNF (Fig. 3E), although PDTC inhibited the activity of NF-κB, induced by BDNF, assessed by the levels of p65 in the nuclear fractions in HCEM cells (data not shown).

Translocation of ERK1/2 from the Cytoplasm to Nucleus in HCEM Cells Following BDNF Stimulation—Incremental z-series images indicated that ERK1/2 was uniformly distributed throughout the cytoplasm and nucleus in unstimulated HCEM cells (Fig. 4A). In contrast, ERK1/2 accumulated in the nucleus in HCEM cells stimulated by BDNF (Fig. 4B). Inhibition of the activation of ERK1/2 with PD98059 blocked the BDNF-induced nuclear translocation of ERK1/2 (Fig. 4C). In addition, transfection of TRKB siRNA resulted in the translocation of ERK1/2 to the nucleus by BDNF being inhibited (Fig. 4D). In contrast, transfection of the negative control siRNA did not influence the nuclear translocation of ERK1/2 (Fig. 4E). The results from the immunofluorescence microscopy were supported with immunoblotting of ERK1/2 in the nuclear fractions and cytoplasmic fractions in HCEM cells (Fig. 4F).
DISCUSSION

In this study, HCEM cells expressed TrkB and p75<sub>NTR</sub>. Furthermore, TrkB and p75<sub>NTR</sub> were not only separately distributed but also partially co-localized in HCEM cells. These results are consistent with the findings that neurotrophin receptors form three different types of complexes as follows: homodimers of trk receptors, a mixed complex of Trk and p75NTR, and homomeric p75NTR receptors (6, 27). Although the stoichiometry of the mixed complex is not well understood, much research into the implications for the Trk-p75<sub>NTR</sub> interaction has been performed (28). Regarding the relationship between the actions of TrkB and p75<sub>NTR</sub> in cells stimulated with BDNF, co-expression of p75<sub>NTR</sub> with TrkB results in a clear increase in the specificity of activation of TrkB by BDNF (29). Conversely, other experiments in vitro have shown that p75<sub>NTR</sub> decreases the phosphorylation of TrkB when induced by BDNF (30). This study using siRNAs for TrkB and p75<sub>NTR</sub> revealed that TrkB signaling induced by BDNF, but not p75<sub>NTR</sub> signaling, is involved in the enhancement of bone/cementum-related protein mRNA expression in HCEM cells. Thus, there may be no interaction between TrkB and p75<sub>NTR</sub> during the increase in bone/cementum-related protein mRNA levels. However, in this study, higher concentrations of BDNF (50 or 100 ng/ml) did not elevate the bone/cementum-related protein mRNA expression, suggesting that a higher concentration of BDNF, but not 20 ng/ml BDNF, activates p75<sub>NTR</sub>, and the p75<sub>NTR</sub> signaling inhibits the increase elicited by TrkB signaling. Additional studies will be required to clarify the link between TrkB and p75<sub>NTR</sub> in HCEM cells stimulated by BDNF.

Previous studies have shown that BDNF protects hippocampal neurons from glutamate-induced cell death or mediates neuromodulation in spinothalamic tract neurons via activation of ERK1/2 (31–33). This study has shown that the activation of ERK1/2 through TrkB is involved in the increase in bone/cementum-related protein mRNA expression caused by BDNF in HCEM cells. Thus, the ERK1/2 signaling pathway mediated by BDNF plays a critical role in activating various cell functions. Activated ERK1/2 is translocated from the cytoplasm to the nucleus (34, 35) and phosphorylates transcription factors such as Elk-1, Myc, and c-Fos (36, 37). Elk-1 is one of the Ets family transcription factors and binds DNA through the helix-turn-helix motif of its Ets domain. Its main function is to regulate expression of the growth-related proteins in response to extracellular stimuli. The binding of a ligand to the tyrosine kinase receptor results in the activation of the proto-oncogene product Ras (38), and the activated Ras phosphorylates c-Raf at Ser-338, suggesting that BDNF stimulates phosphorylation of Elk-1 through the Ras-c-Raf-MEK-ERK1/2 pathway, a process known as canonical MAPK signaling, to enhance bone/cementum-related protein mRNA expression in HCEM cells. In addi-
tion, BDNF binds to TrkB to induce canonical MAPK signaling in HCEM cells.

The OPN promoter contains three consensus T-cell factor-binding sites, three Ets-binding sites, and one AP-1-binding site (41). The T-cell factor-binding sites have been shown to promote the transcription of OPN when bound by the T-cell factor/Elk-1 protein activated by phosphorylation of ERK in fibroblasts, epithelial cells, and T-cells (42, 43). In addition, a previous study had shown that the activation of Elk-1 is involved in the expression of ALP mRNA in human dental pulp cells (44). In this study, the knockdown of ELK-1 completely abolished the increase in OPN and ALP mRNA expression to the basal level. The activation of Elk-1 is thought to elicit the increase in OPN and ALP mRNA levels in human cementoblast cells. On the other hand, knockdown of ELK-1 partially abolished the increase in BMP-2 mRNA levels, suggesting that other transcription factors in addition to Elk-1 are involved in the increase. Because the nuclear transcription factors cAMP-response element-binding protein and STAT-3, which are located downstream of ERK1/2 (45, 46), activate osteoblast differentiation (47–49), cAMP-response element-binding protein or STAT3 as well as Elk-1 may be the transcription factors involved in the enhancement of BMP-2 mRNA expression in HCEM cells stimulated by BDNF.

Inhibition of the ERK1/2 cascade promoted the formation of bone in a mouse osteoblast-like cell line derived from neonatal calvaria, MC3T3-E1 (50), and in a mouse myoblastic cell line, C2C12 (50, 51). In contrast, activation of the ERK1/2 pathway induced osteoblast differentiation in the mouse mesenchymal progenitor cell line C3H10T1/2 (52) and in MC3T3-E1 cells (53). Thus, the physiological role of the ERK1/2 pathway in osteoblasts is still controversial (54). An ERK1/2 inhibitor suppressed the increase in OPN mRNA levels caused by amelogenin peptide in immortalized mouse cementoblasts (55). This study showed that the ERK1/2 pathway in human cementoblasts is involved in the stimulation of ALP, OPN, and BMP-2 mRNA expression and calcification by BDNF, suggesting that ERK1/2 stimulates the differentiation of cementoblasts, independent of stimuli and the source of cementoblasts.

Other growth factors have been examined for their role in the regeneration of periodontal tissue defects (56). Notably, the application of fibroblast growth factor (FGF)-2 enhanced the regeneration of periodontal tissue (57–59). FGF-2 activates ERK1/2 via the FGF receptor (60). Although it remains unknown how FGF-2 influences cementoblast functions, FGF and BDNF, in general, inhibit and stimulate the expression of bone/cementum-related proteins, respectively, in cells forming mineralized tissue, such as osteoblasts, chondrocytes, and periodontal ligament cells (8, 10, 61, 62). Therefore, the...
role of ERK1/2 activated by FGFR-2 and BDNF in the regeneration of periodontal tissue may be different.

In conclusion, BDNF activates a TrkB-c-Raf-ERK1/2 signaling pathway in HCEM cells. The culmination of these signaling events is the up-regulation of the mRNA expression of ALP, OPN, and BMP-2 (Fig. 7). These observations provide critical new insights into the mechanisms whereby BDNF promotes the regeneration of periodontal tissue and suggest that ERK1/2 signaling contributes to the enhancement in the formation of mineralized tissues, such as cementum and bone.

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REFERENCES

1. Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., and Chao, M. (1986) Cell 47, 545–554
2. Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., and Shooter, E. M. (1987) Nature 325, 593–597
3. Ebendal, T. (1992) J. Neurosci. Res. 32, 461–470
4. Barbacid, M. (1994) J. Neurobiol. 25, 1386–1403
5. Bibel, M., and Barde, Y. A. (2000) Curr. Opin. Neurobiol. 10, 374–381
6. Otten, U., Ehrhard, P., and Peck, R. (1989) J. Cell Biol. 109, 593–597
7. El-Tanani, M., Fernig, D. G., Barraclough, R., Green, C., and Rudland, P. (1986) J. Biol. Chem. 261, 1618–1629
8. Kurihara, H., Shinohara, H., Yoshino, H., and Ishihara, K. (2000) Tissue Eng. 11, 1618–1629
9. Kursun, O., Kuchler, K., Lohmann, M., Jooss, B., Hönisch, M., and Rall, K. (2000) J. Periodontol. 71, 616–622
10. Debiais, F., Hott, M., Graulet, A. M., and Marie, P. J. (1998) J. Bone Miner. Res. 13, 1671–1678
11. Nakayama, K., Tamura, Y., Suzawa, M., Harada, S., Fukumoto, S., Kato, M., Miyazono, K., Rodan, G. A., Takeuchi, Y., and Fujita, T. (2003) J. Biol. Chem. 278, 21951–21958
12. Nosrat, C. A., Fried, K., Lindskog, S., and Olson, L. (1997) J. Biol. Chem. 272, 14553–14556
13. Schlessinger, J., and Bar-Sagi, D. (1994) Cold Spring Harbor Symp. Quant. Biol. 59, 173–179
14. Otten, U., Ehrhard, P., and Peck, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 10059–10063
15. MacGrogan, D., Saint-Andre, J. P., and Dicou, E. (1992) J. Neurosci. Res. 31, 1381–1391
16. Yamashiro, T., Fukunaga, T., Yamashita, K., Kobaishi, N., and Takano-Yamamoto, T. (2001) Bone (N.Y.) 28, 404–409
17. Kitagawa, M., Kaida, M., Kario, K., Hino, T., Kado, S., Ogawa, I., Miyai, M., and Takata, T. (2006) Bone (N.Y.) 39, 1035–1042
18. El-Tanani, M., Fernig, D. G., Barraclough, R., Green, C., and Rudland, P. (1986) J. Biol. Chem. 261, 1618–1629
19. Kurihara, H., Shinohara, H., Yoshino, H., Takechi, Y., and Shiba, K. (2003) J. Periodontol. 74, 76–84
20. Nosrat, C. A., Fried, K., Lindskog, S., and Olson, L. (1997) Cell Tissue Res. 290, 569–580
21. Furukawa, Y., Furukawa, S., Satoyoshi, E., and Hayashi, K. (1984) J. Biol. Chem. 259, 1259–1264
22. Otten, U., Ehrhard, P., and Peck, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 10059–10063
23. MacGrogan, D., Saint-Andre, J. P., and Dicou, E. (1992) J. Neurochem. 59, 1381–1391
24. Xiao, G., Gopalakrishnan, R., Jiang, D., Reith, E., Benson, M. D., and Franceschi, R. T. (2002) J. Bone Miner. Res. 17, 101–110
25. Chen, C., Koh, A. J., Datta, N. S., Zhang, J., Keller, E. T., Xiao, G., Franceschi, R. T., D’Silva, N. J., and McCauley, L. K. (2004) J. Biol. Chem. 279, 29211–29219
26. Takeuchi, Y., Suzawa, M., Kikuchi, T., Nishida, E., Fujita, T., and Matsusato, T. (1997) J. Biol. Chem. 272, 29309–29316
27. Xiao, G., Jiang, D., Gopalakrishnan, R., and Franceschi, R. T. (2002) J. Biol. Chem. 277, 36181–36187
28. You, J., Reilly, G. C., Zhen, X., Yellowley, C. E., Chen, Q., Donahue, H. J., and Jacobs, C. R. (2001) J. Biol. Chem. 276, 13365–13371
29. Dahl, L. K. (1952) Proc. Soc. Exp. Biol. Med. 80, 474–479
30. Jang, S., Tapley, P., and Barbacid, M. (1992) Neuron 9, 1067–1079
31. Huang, E. J., and Reichardt, L. F. (2003) Annu. Rev. Biochem. 72, 609–642
32. Bibel, M., Hoppe, E., and Barde, Y. A. (1999) EMBO J. 18, 616–622
33. Vesa, J., Krüttgen, A., and Shooter, E. M. (2000) J. Biol. Chem. 275, 24414–24420
34. Takeuchi, Y., Suzawa, M., Harada, S., Fukumoto, S., Kato, M., Miyazono, K., Rodan, G. A., Takeuchi, Y., and Fujita, T. (2003) J. Bone Miner. Res. 18, 827–835
35. Higuchi, C., Myoui, A., Hashimoto, K., Kuriyama, K., Yoshikawa, K., Yoshihara, H., and Itoh, K. (2002) J. Bone Miner. Res. 17, 1785–1794
36. Lou, J., Yu, T., Li, S., and Manske, P. R. (2000) Biochem. Biophys. Res. Commun. 268, 757–762
37. Xiao, G., Jiang, D., Thomas, P., Benson, M. D., Guan, K., Karsenty, G., and Franceschi, R. T. (2000) J. Biol. Chem. 275, 4453–4459
38. Schindeler, A., and Little, D. G. (2006) J. Bone Miner. Res. 21, 1331–1338
39. Bouabdall, T., and Fratzl, P. (2003) J. Bone Miner. Res. 18, 1126–1136
40. Derycke, X. E., Markopoulos, C. E., and Vrotsos, I. A. (2006) Growth Factors 24, 260–267
41. Murakami, S., Takayama, S., Ikezawa, K., Shimabukuro, Y., Kitamura, M., Nozaki, T., Terashima, A., Asano, T., and Okada, H. (1999) J. Periodontal Res. 34, 425–430
42. Murakami, S., Takayama, S., Ikezawa, K., Shimabukuro, Y., Yanagi, K., Ikezawa, K., Saho, T., Nozaki, T., and Okada, H. (2003) J. Periodontal Res. 38, 97–103
43. Sato, Y., Kikuchi, M., Ohata, N., Tamura, M., and Kuboki, Y. (2004) J. Periodontol. 75, 243–248
44. Wang, J. K., Gao, G., and Goldfarb, M. (1994) Mol. Cell. Biol. 14, 181–188
45. Kato, Y., and Iwamoto, M. (1990) J. Biol. Chem. 265, 5903–5909
46. DeBiasi, F., Hott, M., Graulet, A. M., and Marie, P. J. (1998) J. Bone Miner. Res. 13, 645–654