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Effects of epidermal growth factor and/or nerve growth factor on Malassez's epithelial rest cells *in vitro*: expression of mRNA for osteopontin, bone morphogenetic protein 2 and vascular endothelial growth factor

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Background and Objective: Malassez's epithelial rest (MER) cells are involved in the maintenance and homeostasis of the periodontal ligament (PDL). The purpose of this study was to determine the effects of epidermal growth factor (EGF) and/or nerve growth factor (NGF) *in vitro* on these functions of MER cells.

Material and Methods: MER cells from porcine PDL were incubated for 3 or 9 h after the addition of EGF and/or NGF to final concentrations of 10 ng/mL. Cells cultured without those growth factors were used as controls. The expression of mRNA for osteopontin, bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF) was analyzed using quantitative RT-PCR.

Results: There was a decrease in the expression of osteopontin mRNA by MER cells treated for 9 h with NGF and the level of mRNA expressed was lower than that of the control and EGF-treated groups. The expression of BMP-2 mRNA by MER cells treated with NGF for 9 h also decreased, and was lower than that of the control and EGF-treated groups. The expression of VEGF mRNA by MER cells treated with EGF for 3 or 9 h was higher than in the control and NGF-treated groups. The expression of VEGF mRNA was lower in MER cells treated with NGF for 3 and 9 h than in the control and EGF-treated groups, and decreased from 3 to 9 h of treatment. EGF stimulated MER cells to secrete VEGF, which suggests that EGF plays an important role in maintaining the homeostasis of the PDL. NGF acts on MER cells to inhibit calcification in the

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PDL. Furthermore, in the EGF + NGF-treated MER cells, expression of mRNA for BMP-2 and VEGF was similar to that of the NGF-treated group, but cell proliferation and expression of osteopontin mRNA were similar to that of the EGF-treated group.

Conclusion: EGF and NGF play important roles in maintaining the PDL.

Malassez's epithelial rest (MER) cells are clusters of epithelial cells derived from Hertwig's epithelial root sheath (1). MER cells form a network of epithelial strands throughout the periodontal ligament (PDL) and are usually found in the inner zone of the PDL near the tooth root cementum (2,3). They appear to be unique with respect to their ability to persist throughout life deep within a connective tissue (4). MER cells are characterized by condensed rounded nuclei with a high nuclear/cytoplasmic ratio, Golgi complexes accompanied by vesicles and a poorly developed rough endoplasmic reticulum in the cytoplasm (5). MER cells have a number of distinct functions, such as to prevent resorption of the root (6), to participate in elongation of the periodontal pocket (7) and periapical cyst formation (8), to induce cementum formation (9) and to maintain the homeostasis of the PDL (10,11). Although many reports have been published on the functional roles of MER cells, including those cited above, there is insufficient evidence to fully elucidate their various functional roles.

MER cells in uninflamed PDL appear to be quiescent and do not show mitotic activity (12). However, it is known that because inflammation occurs in the PDL, MER cells can be stimulated to proliferate (13). Furthermore, it has been suggested that growth factors, such as epidermal growth factor (EGF), may play an important role in regulating MER cell proliferation, and that EGF acts not only on epidermal cell proliferation but has several other cellular functions. EGF is a small, hormone-like polypeptide that was isolated from mouse submandibular glands and has the ability to accelerate incisor eruption and eyelid opening (14). When inflammation occurs in the PDL, Harris & Toller (15) reported that MER cells begin to proliferate, and Nordlund *et al*. (16) suggested that EGF affects MER cells.

Neuroendocrine cells in the MER may support the periodontal nerve supply and act as receptors in the mechanical transmission of stimuli in the PDL (17). Nerve growth factor (NGF) plays crucial roles during the normal development, differentiation and survival of neurons in the central and peripheral nervous systems. When neurons are injured, NGF is capable of supporting their survival and stimulates the neurite outgrowth needed for neuronal regeneration (18). MER cells are known to be immune-positive for TrkA, a high-affinity NGF receptor, and denervation of the inferior alveolar nerve results in a marked decrease in the distribution and size of MER cell clusters (19–21), which leads to dento–alveolar ankylosis with a decrease in the width of periodontal spaces. However, the width of the PDL shows a correspondingly significant increase with the regeneration of MER cells after the denervation (22). Furthermore, NGF is expressed during the healing of bone fractures and is involved in nerve maintenance in intact bone as well as in the upkeep and proliferation of nerve fibers during bone repair (23). These findings suggest that the sensory nerve could play a regulatory role in maintaining MER cells and that MER cells may be involved in the maintenance of the periodontal space.

The purpose of this study was to investigate the effects of EGF and/or NGF on the maintenance and homeostatic functions of MER cells on the PDL.

Material and methods

Cell culture

MER cells from porcine PDL were provided by Professor Yoshihiro Abiko (Department of Dental Science, Institute of Personalized Medical Science, Health Science University of Hokkaido, Hokkaido, Japan). The method for obtaining MER cells has been described previously by Liu *et al*. (24). The culture medium used for MER cells was alpha-minimal essential medium (α-MEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma, St Louis, MO, USA) and gentamycin (Sigma). The MER cells were suspended and cultured in 75-cm^2 tissue culture flasks (Corning, Tokyo, Japan) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. When the cells were almost confluent, they were detached using 0.25% trypsin/0.02% EDTA (pH 7.2). Then, the cells were resuspended in the supplemented culture medium described above and used for the experiments.

Identification of epithelial cells and the presence of receptors for EGF and NGF

To identify epithelial cells, immunofluorescence staining was carried out using a primary antibody against cytokeratin 19 (1:100 dilution; Abcam, Cambridge, UK), a secondary antibody labeled with rhodamine (1:100 dilution; Invitrogen, Carlsbad, CA, USA) and 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen™). Briefly, after the primary culture, cells were fixed with 10% formalin for 10 min. After immersion in 1% Triton X-100 for 5 min, the cells were incubated with the primary antibody for cytokeratin 19 and then with the secondary antibody labeled with

rhodamine and with DAPI. The cells were then observed using a confocal laser scanning microscope (LSM 5 DUO; Carl Zeiss, Jena, Germany).

Western blotting was carried out according to the method of Amemiya *et al.* (25). Briefly, MER cells were lysed in radio-immunoprecipitation assay buffer containing inhibitors. Twenty-five micrograms of protein in each total lysate was subjected electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (BioRad, Hercules, CA, USA). The membranes were then incubated overnight with anti-cytokeratin 19 (44 kDa, 1:500 dilution; Abcam) at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated rabbit IgG (1:1000 dilution; Amersham, Rochester, MI, USA) at room temperature (20°C) for 1 h. Immunoreactive bands were detected using an Immun-Blot assay kit (BioRad).

Approximately 1.0×10^5 MER cells were seeded into each well of six-well Multiwell dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 3 or 9 h after the addition of EGF and/or NGF to final concentrations of 10 ng/mL. The concentrations of NGF and EGF used in the present study were based on the concentrations used in previous studies carried out by Xu *et al.* (26) and Matsuda *et al.* (27). Cells cultured without growth factors were used as controls. The culture medium was not changed during the course of the experiment.

To confirm the effects of EGF and/or NGF on MER cells, immunofluorescence staining was also carried out to detect the presence of receptors for both growth factors using antibodies against EGF receptor (EGF-R) (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TrkA (1:50 dilution; Santa Cruz Biotechnology), followed by secondary antibodies labeled with rhodamine or fluorescein isothiocyanate (FITC) (1:100 dilution; Invitrogen) along with DAPI staining. Cells were observed using confocal laser scanning microsopy (CLSM). Experiments performed to confirm the presence of receptors were repeated three times.

Cell adhesion observations

MER cells were cultured with or without 10 ng/mL of NGF or EGF on 15-mm cell disks, and were observed using immunofluorescence microscopy at 3 h and at 24 h of culture. The cells were stained using an anti-P-FAK (phospho Y397) immunoglobulin (1:100 dilution; Abcam) and a vinculin antibody (1:100 dilution; Chemicon, Temecula, CA, USA) using the same methods described above for cytokeratin 19. The cells were then observed using CLSM. Cell-adhesion experiments were repeated three times.

Cell proliferation assay

Cells were seeded at a density of approximately 1.0×10^5 cells per well in 24-well plates (Falcon) and were cultured. The cells were detached at 3 h and at 24 h of culture, and on days 3 and 5 of culture, using trypsin/EDTA after the addition of 10 ng/mL of NGF and/or EGF, or without any growth factor as a control, and were counted using a Coulter counter (Coulter Z-1; Coulter, Tokyo, Japan) at each time-point. Cell proliferation assay experiments were reproduced four times.

Quantitative RT-PCR

Total RNA was extracted using the acid guanidium thiocyanate/phenol–chloroform method as follows. After 3 or 9 h of treatment, the culture medium of each dish was removed and the cells were rinsed twice with phosphate-buffered saline. The cells were homogenized in 1 mL of TRIsol Reagent (Invitrogen) and each solution was transferred to a 1.5 mL tube containing chloroform and was mixed. Each tube was centrifuged at 16,110 ***g*** for 20 min at 4°C, after which each supernatant was transferred to a 1.5 mL tube containing 250 μL of 100% isopropanol (one-quarter of the volume of TRIsol Reagent) and incubated at −80°C for 1 hr. After centrifugation at 16,110 ***g*** for 20 min at 4°C, the supernatants were discarded and the remaining total RNA pellets were washed with 70% cold ethanol. The total RNA pellets were dissolved in 50 μL of RNAse-free (Diethyl Pyrocarbonate-treated) water. Total RNA was reverse transcribed and amplified in 20-μL volumes using a Reverse Transcription Kit (Quanti Tect; Qiagen, Germantown, MD, USA) containing RNA PCR Buffer, 2 U/μL of RNAse inhibitor, 0.25 U/μM reverse transcriptase, 0.125 μM oligo dt-adaptor primer, 5 mM $MgCl_2$ and RNAse-free water. The RT-PCR products were analyzed using quantitative real-time RT-PCR in TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for the target genes bone morphogenetic protein 2 (BMP-2), osteopontin and vascular endothelial growth factor (VEGF). The TaqMan Endogenous Control (Applied Biosystems) for the target gene beta-actin was used as a control. The primer sequences used are shown in Table 1. All PCR reactions were performed using a real-time PCR 7500 fast system. Quantification of gene expression using TaqMan Gene Expression Assays was performed as the second step in a two-step RT-PCR. Assays were performed in 20-μL singleplex reactions containing TaqMan Fast Universal PCR Master Mix, TaqMan Gene Expression Assays, distilled water and complementary DNA (cDNA), according to the

Table 1. Primer sequences

Primer	Genbank	Forward	Reverse	Probe
β-actin	DQ845171	CTCCCTGGAGAAGAGCTACGA	AGCGGAAGCGCTCGTT	ACGGCCAGGTCATCAC
OPN	X6575	CCCAAGGCCATCCTCGTT	GTCTCCTGACTGTCCTTCTCTTG	CCCAGCGCCTGCACG
VEGF	AF318502	CATCTTCAAGCCGTCCTGTGT	CCAGACCTTCGTCGTTGCA	CCCGCACCGCATCAG
BMP2	AY669080	TCAGCAGAACTTCAGGTCTTTCG	CGGTGATGGAAACTGCTACTGTTAT	CAGACACAGGAGACTTT

manufacturer's instructions (Applied Biosystems). The reaction conditions consisted of a primary denaturation at 95°C for 20 s, then cycling for 40 cycles at 95°C for 3 s and 62°C for 30 s. PCR data were reported compared with the corresponding control. Quantitative RT-PCR analyses were reproduced four times.

Statistical analysis

Data were analyzed using one-way analysis of variance and were compared using Scheffe's test.

Results

Immunofluorescence observations of cytokeratin 19, EGF-R and TrkA

MER cells were stained for cytokeratin 19 using an immunofluorescence technique (Fig. 1A). Western blot analysis showed that cytokeratin 19 was expressed constitutively by the MER cells (Fig. 1B). The MER cells were immunopositive for EGF-R and TrkA (Fig. 2A,B).

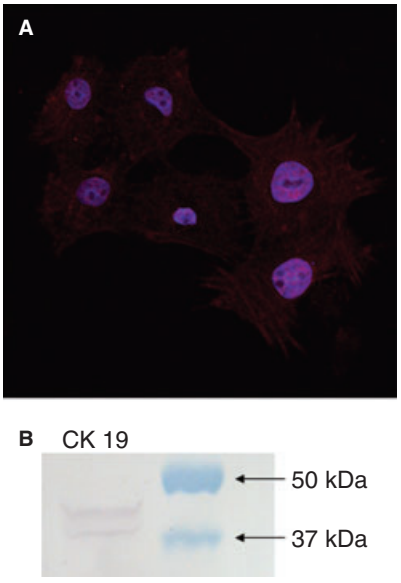

Fig. 1. (A) Immunofluorescence image of Malassez's epithelial rest (MER) cells using an antibody to cytokeratin 19 (CK 19). (B) Western blot analysis with an antibody to CK 19. The cells used in this study were confirmed as MER cells by immunofluorescence and by western blotting.

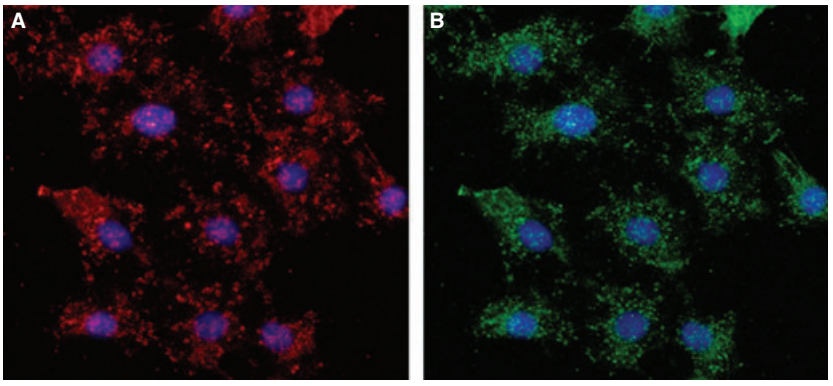

Fig. 2. Confocal laser scanning microscopy (CLSM) images of Malassez's epithelial rest (MER) cells stained with antibodies to the epidermal growth factor (EGF) receptor (A) and to TrkA (B) after 1 d of culture. Note that the EGF and nerve growth factor (NGF) receptors are expressed by MER cells.

Immunofluorescence observations of P-FAK and vinculin

MER cells were immunopositive for P-FAK after 3 h of treatment with EGF, but were not immunopositive for P-FAK after 24 h of treatment with EGF. MER cells treated for 3 h with NGF, and the control MER cells, were only slightly immunopositive for P-FAK, but after 24 h, neither group of cells was immuno-positive for P-FAK. By contrast, MER cells treated with EGF or NGF for 3 or 24 h were immunopositive for vinculin, as were the control cells (Fig. 3).

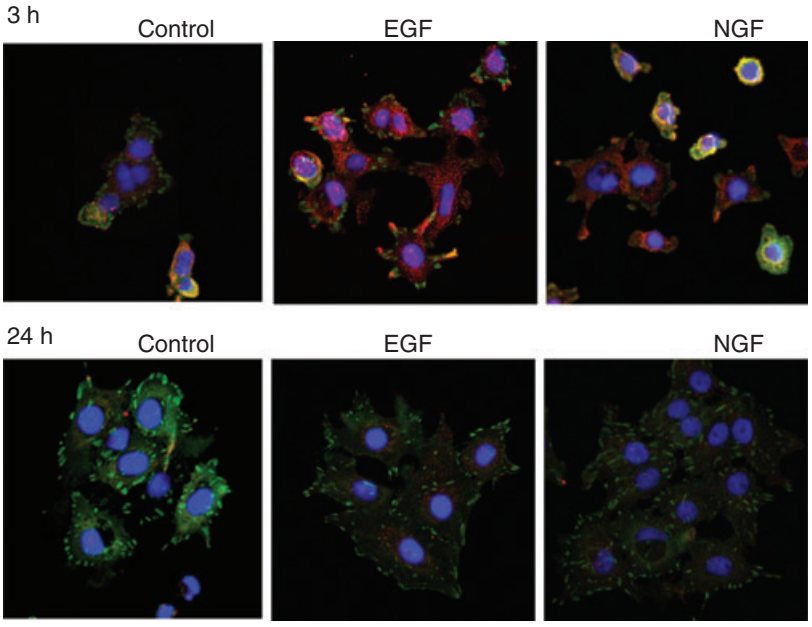

Fig. 3. Confocal laser scanning microscopy (CLSM) images of Malassez's epithelial rest (MER) cells stained with antibodies to P-FAK and to vinculin after 3 or 24 h of treatment with the indicated factors. Red, P-FAK; green, vinculin. MER cells treated with epidermal growth factor (EGF) for 3 h were immunopositive for P-FAK. MER cells treated with nerve growth factor (NGF), and the untreated control cells, were only slightly immunopositive for P-FAK, but after 24 h were not positive for P-FAK. Vinculin was immunopositive in MER cells treated with EGF or NGF, and in the untreated control cells, at both 3 and 24 h.

Cell proliferation assays

The numbers of MER cells increased during the course of the experiment, regardless of whether they were, or were not, treated with EGF and/or NGF. MER cells treated with EGF for 3 d and for 5 d showed a significantly higher rate of growth than cells in control and the NGF-treated groups (Fig. 4). By contrast, MER cells treated with both EGF and NGF showed a rate of growth similar to that of MER cells treated with EGF alone.

Expression of mRNA for osteopontin, BMP-2 and VEGF

The expression of osteopontin mRNA by MER cells treated with NGF for 9 h decreased, and was lower than that of the control and the EGF-treated groups. Furthermore, the expression of osteopontin mRNA by MER cells treated with both EGF and NGF for 9 h was similar to that of MER cells treated with EGF alone (Fig. 5). The expression of BMP-2 mRNA by MER cells treated with EGF or NGF for 9 h also decreased, and was lower than that of the control group. The expression of BMP-2 mRNA by MER cells treated with both EGF and NGF for 9 h was similar to that of the group treated with NGF alone (Fig. 6). The expression of VEGF mRNA by MER cells treated with EGF for 3 or 9 h was higher than that found in control and NGF-treated groups, and the expression of VEGF mRNA by cells treated with NGF for 3 or 9 h was lower than found in control and EGF-treated groups. Furthermore, the expression of VEGF mRNA by MER cells treated with NGF decreased from 3 to 9 h of treatment. The expression of VEGF mRNA by MER cells treated with both EGF and NGF for 3 and 9 h was similar to that in the NGF-treated group (Fig. 7).

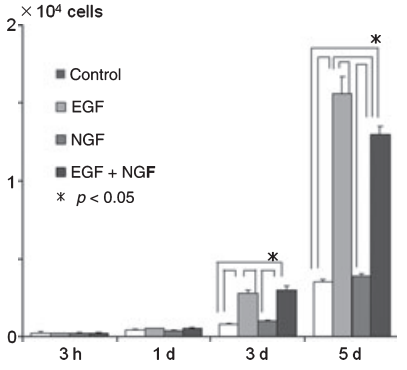

Fig. 4. Cell numbers. The numbers of Malassez's epithelial rest (MER) cells treated with epidermal growth factor (EGF) for 3 d and for 5 d were significantly higher than the numbers of control cells and those treated with nerve growth factor (NGF), alone for 3 d and 5 d, and higher than the number of MER cells treated with EGF + NGF on day 5 only, $*p < 0.01$.

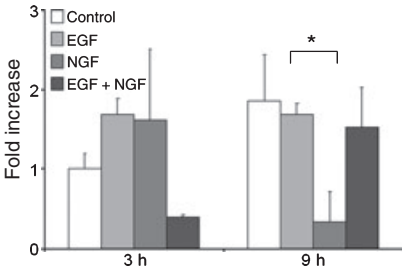

Fig. 5. Expression of osteopontin mRNA. There was no significant difference between the epidermal growth factor (EGF)-treated group and the control group at 3 or 9 h. Expression of osteopontin mRNA by Malassez's epithelial rest (MER) cells treated with nerve growth factor (NGF) for 9 h was significantly lower than in the control group and showed a decrease from 3 to 9 h of treatment, $*p < 0.01$.

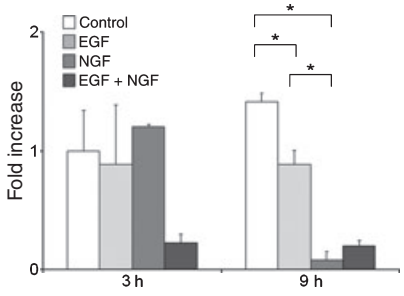

Fig. 6. Expression of bone morphogenetic protein 2 (BMP-2) mRNA. Expression of BMP-2 mRNA by Malassez's epithelial rest (MER) cells treated with epidermal growth factor (EGF) or nerve growth factor (NGF) for 9 h was lower than in the control group. Expression of BMP-2 mRNA in the NGF-treated group decreased from 3 to 9 h of treatment, $*p < 0.01$.

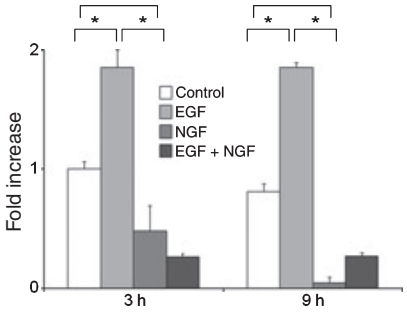

Fig. 7. Expression of vascular endothelial growth factor (VEGF) mRNA. Expression of VEGF mRNA by Malassez's epithelial rest (MER) cells treated with epidermal growth factor (EGF) for 3 or 9 h was higher than in the control cells and in the cells treated with nerve growth factor (NGF). Expression of VEGF mRNA in cells treated with NGF for 3 or 9 h was lower than in the control cells and in the cells treated with EGF. Expression of VEGF mRNA by MER cells treated with NGF decreased from 3 to 9 h of treatment, $*p < 0.01$.

Discussion

Many studies have been published on the various functional roles of MER cells, and MER cells are known to be involved with homeostasis of the PDL. Various growth factors affect MER cells, and in this study we studied the influences of EGF and/or NGF on MER cells to determine their effects on the maintenance and homeostasis of the PDL. MER cells derived from porcine PDL were used, and their identity was confirmed by immunoreactivity for cytokeratin 19 using immunofluorescence and western blotting.

EGF is a small (6 kDa) single-chain polypeptide and is a potent mitogen for many cell types, including epithelial cells. EGF stimulates the proliferation and keratinization of cells in the oral epithelium (27). EGF is an important growth factor that elicits cellular signaling through an EGF receptor and affects the migration of many types of cells (27). After epithelial injury, EGF-R expression is up-regulated in migrating and proliferating keratinocytes adjacent to the wound. Fujita *et al.* (28) previously reported that EGF down-regulates alkaline phosphatase activity in PDL cells. Lindskog *et al.* (11) showed that the functions of

EGF include maintaining the PDL space and preventing dento–alveolar ankylosis. Those studies suggest that EGF plays a similar role, not only in PDL cells but also in MER cells with respect to calcification. By contrast, NGF is known to be secreted from nerve tissues to expedite healing after injury. Yamashiro *et al*. (19) reported the results of an *in vivo* study showing that MER cells express a high-affinity NGF receptor. In our *in vitro* study, the expression of EGF receptor and NGF receptor by MER cells was demonstrated using immunofluorescence microscopy. The concentrations of NGF and EGF used were 10 ng/mL, based on studies of the dose-dependent effects of NGF (26) and of EGF (27). A 10 ng/mL concentration of EGF and NGF was used in this study because these were found to be the minimum effective concentrations of EGF and NGF in those previous studies.

Cell-to-substrate adhesion plays an important role in cell proliferation, cell differentiation and expression of other cellular functions. FAK and vinculin are known as important adherence proteins involved in cell-to-substrate adhesion. FAK plays a key role in the crosstalk of the growth factor- and cell adhesion-mediated signaling pathways. In adherent cells, FAK colocalizes with integrins at the sites of cell–matrix contacts termed focal adhesions (29,30). MER cells treated with EGF were highly immunopositive for P-FAK compared with the control and NGF-treated groups. Furthermore, in this study, the number of MER cells in the EGF-treated group was higher than in the control and NGF-treated groups. Thus, EGF acts on MER cells to promote their initial adhesion to the substrate and cell proliferation. A previous study demonstrated that EGF-R-induced signaling pathways are necessary for cell migration (31,32). The number of MER cells treated with both EGF and NGF was similar to that of the group treated with EGF alone. These results show that EGF stimulates MER cell proliferation, but NGF does not.

Osteopontin is known to participate in cementum formation (33). MER cells secrete osteopontin during the early stages of cementum repair *in vivo* (34) and osteopontin mRNA is expressed by cultured MER cells (35). Xu *et al*. (26) reported that the expression of osteopontin mRNA by PDL cells increased in a dose-dependent manner following treatment with NGF. BMP-2 is also well known for its involvement in calcification of the matrix, and it was recently reported that MER cells express BMP-2. Xu *et al*. (26) further showed that the expression of BMP-2 mRNA by PDL cells was increased by treatment with NGF. In the present study, NGF acted by decreasing the expression of osteopontin and BMP-2 mRNAs by MER cells, which means that NGF causes MER cells to inhibit osteogenesis. There was no significant difference in expression of osteopontin mRNA between the control cells and the cells of the EGF-treated group. Moreover, the expression of BMP-2 mRNA by MER cells treated with both EGF and NGF for 9 h was similar to the expression of BMP-2 mRNA by MER cells treated with NGF alone, but expression of osteopontin mRNA by MER cells treated with both EGF and NGF was similar to that of the EGF-treated group. Although osteopontin is not only involved with calcification but has other varied functions, BMP-2 is known for its role in calcification. Thus, it is thought that NGF works particularly well with EGF on MER cells with respect to calcification.

VEGF functions as an angiogenic growth factor that elicits cellular responses to stimulation (36,37), and is a multifunctional cytokine that contributes to angiogenesis via direct and indirect mechanisms (38). The development of endothelial cell-based microvascularization and microcirculation is undoubtedly crucial for the preservation of structure and regeneration (39). Immunohistochemically, VEGF is expressed not only in inflamed epithelium but also in the MER (40). In this study, the expression of VEGF mRNA by MER cells was up-regulated by EGF. This means that not only may EGF stimulate the proliferation of MER cells but also suggests that the secretion of VEGF by MER cells stimulates proliferation further. In this study, expression of VEGF mRNA by MER cells treated with NGF was lower than found in the untreated control. Campos *et al*. (41) reported that NGF up-regulates VEGF expression in epithelial ovarian cancer cells. However, in this study, expression of VEGF mRNA by MER cells was down-regulated by NGF, which suggests that NGF plays a role in MER cells to inhibit hypervascularization. The expression of VEGF mRNA by MER cells treated with both EGF and NGF for 3 and 9 h was similar to that of the NGF-treated group. Thus, NGF seems to work particularly well with EGF for MER cells.

In conclusion, while injurious stimulation elicits the secretion of the growth factors EGF and NGF, EGF affects MER cells by increasing the expression of VEGF mRNA, while NGF causes MER cells to down-regulate their expression of osteopontin and BMP-2 mRNAs. Thus, EGF and NGF may cooperate to modulate MER cell function needed to maintain the PDL.

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