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A mineralization-associated membrane protein plays a role in the biological functions of the peptide-coated bovine hydroxyapatite

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Background and Objective: Anorganic bovine mineral coated with a cell-binding peptide (P-15) is superior to anorganic bovine mineral alone in the treatment of periodontal osseous defects. However, the molecular interactions between P-15 and periodontal ligament fibroblasts remain unclear.

Material and Methods: We first compared the *in vitro* osteogenic activities between periodontal ligament fibroblasts cultured with anorganic bovine mineral alone and with the P-15/anorganic bovine mineral combination. We then harvested the periodontal ligament cell lysate, incubated it with various graft materials, and then washed it to remove unbound proteins. The bound proteins were eluted from graft materials and analyzed using electrophoresis, followed by mass spectrometry and then western blotting. Finally, a neutralizing antibody against one bound protein was added to the cell cultures to repeat the osteogenic assays to clarify its role in the *in vitro* effects of the P-15/anorganic bovine mineral combination.

Results: Cells treated with P-15/anorganic bovine mineral were more viable and showed greater osteogenic activities than cells treated with anorganic bovine mineral alone and the no-graft control. Annexin II, a mineralization-associated protein, bound to P-15/anorganic bovine mineral significantly more than to anorganic bovine mineral alone. The addition of neutralizing antibody for annexin II decreased the osteogenic activities of the P-15/anorganic bovine mineral combination.

Conclusion: Annexin II of periodontal ligament fibroblasts interacted with the peptide of P-15, and was partially responsible for better *in vitro* osteogenesis in the P-15 graft.

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Xenogeneic bone grafts are bone grafts derived from the exoskeletons of other species. There are two major sources for xenogeneic bone grafts: bovine bone and natural coral (1). In order to eradicate the immunogenic materials in the xenogeneic skeleton, processing of the materials usually consists of sintering, following chemical deproteinization,

to yield natural hydroxyapatite (2). Depending on the sintering temperatures, these biologic hydroxyapatites are divided into grafts produced at low (< 450°C) or high (> 450°C) temperature (2). Bovine hydroxyapatite produced at low temperature has a higher porosity, larger pores, and larger granules than that produced at high temperature. The larger pores increase the surface area of the graft and provide a better osteoconductive scaffold for osteoblasts to adhere to (3). However, bovine hydroxyapatite produced at low temperature contains residual proteins that might transmit cross-species diseases, such as mad cow disease (bovine spongiform encephalopathy) (4–7). Hydroxyapatites produced at high temperature have significantly smaller pores, but they have no remaining proteins (4). The disadvantage of reduced porosity and smaller pores may be partly compensated for by smaller granules. One of the high-temperature products, produced at a sintering temperature of > 1000°C, can be divided into a further two products (OsteoGraf®/N-300 and N-700; Densply CeraMed Dental, Lakewood, CO, USA) according to particle size. This product has been described as anorganic bovine mineral or matrix that, when used alone or in combination with an allograft, effectively improves bone healing in the sinus floor elevation procedure (8,9).

The adherence of osteoblasts to foreign graft material is a critical step in new bone formation (10). For the cell-binding domain of type I collagen, the major organic component in calcified tissue, a 15-amino-acid peptide, is most putative. A commercial product (PepGen p-15®; Densply CeraMed Dental) has therefore been developed to coat anorganic bovine mineral with this 15-chained peptide to increase the adherence, migration, proliferation, and differentiation of osteoblasts (11–14). Use of this peptide-coated graft in periodontal intrabony defects yields better clinical results than using anorganic bovine mineral alone, demineralized freeze-dried bone allografts, or open-flap debridement (15,16). Although this peptide-coated graft increased the expression of transforming growth factor-β – a cytokine associated with osteodifferentiation – in oral fibroblasts (13,14,17), little information is available about the molecular mechanisms of the biological effects of this product. More molecular information was provided using cDNA microarray methodology in a recent study (18) that treated an osteoblast cell line with this analogue peptide and looked for the downstream molecules. A limited number of genes (some related to the cytoskeleton and some to the extracellular matrix) were up-regulated. Some genes involved in apoptosis and cell-cycle control were down-regulated. However, there is still no molecular information about how the analogue peptide interacts with the cells responsible for periodontal regeneration.

Periodontal ligament fibroblasts are accepted as one of the major contributors to periodontal regeneration (19,20). When a peptide-coated bone graft is placed in a periodontal osseous defect, whether the peptide binds to receptor-like molecules on the cell membrane or enters the cytoplasm directly and initiates the signaling cascade remains largely unknown. We aimed to compare the proliferative and differentiative abilities of the peptide-coated anorganic bovine mineral with anorganic bovine mineral alone, and to identify proteins that can interact with the cell-binding peptide by using mass spectrometry, a current proteomic method used for identifying proteins (21).

Materials and methods

Cell culture of periodontal ligament fibroblasts

Human periodontal ligament (periodontal ligament) fibroblasts were isolated from the extracted third molar of a healthy 20-yr-old man. A signed informed-consent form, previously approved by the Institutional Review Board of our university, was obtained from this patient. The periodontal ligament fibroblasts were cultured according to the method of Somerman *et al.* (22). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 μg/mL), in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells used in this study were within passages three to five.

Cell survival and proliferation assay (MTT assay)

In the following experiments, the graft materials in the experimental groups were peptide-coated anorganic bovine mineral (denoted as P-15) and anorganic bovine mineral of the same particle size (denoted as N-300) as P-15. Approximately 2×10^4 fibroblasts, suspended in 500 μL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, were plated onto 24-well tissue-culture plates, which received no graft, or 10 mg of P-15 or N-300, in each well. After incubation of the fibroblasts for 3 d, the relative number of cells in each sample was determined using the Mosmann protocol (23). At the end of the culture period, 200 μL of a tetrazolium dye complex [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide], at a concentration of 1 mg/mL in Dulbecco's modified Eagle's medium, was added to each well, and the plates were incubated for 4 h at 37°C under 5% CO_2. The solubilization reagent (500 μL of dimethylsulfoxide) was then added to the cell cultures and left for 1 h in a humidified atmosphere. Finally, 150 μL of each cultured solution was transferred to a separate well on a 96-well enzyme-linked immunosorbent assay plate, and the absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad, Sydney, Australia). The samples of each tested group were in replicates of six and the experiment was repeated twice.

Alkaline phosphatase activity

We used two methods to determine alkaline phosphatase activity. One was measuring the alkaline phosphatase activity of the total cell lysate from cells co-incubated with different bone

graft materials. The other was chemically staining the cells cultured on glass slides. Periodontal ligament fibroblasts were plated into 24-well plates, as described above. Each well was filled with 10 mg of either bone graft or no graft, in advance. After 10 d of culture, the cells were rinsed three times in phosphate-buffered saline and frozen to allow the cells to undergo lysis. Five-hundred microliters of 1 mg/mL *p*-nitrophenol phosphate in 0.1 M diethanolamine (pH 8.3) was added to each well and incubated at 25°C for 30 min with gentle agitation. The enzymatic color reaction was stopped by adding 500 μL of 0.75 M NaOH, and the mixture was assayed for absorbance at 405 nm in a microplate reader. The optical density values were normalized with the corresponding MTT values to eliminate the error from different cell numbers. When chemically staining the cells on cover slides, we first placed 10 mg of either bone graft on a sterile glass cover slide (24 × 24 mm) that was in the middle of a 3-cm culture dish. Approximately 5×10^4 fibroblasts, suspended in 200 μL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, were added to the cover slide. After incubating the fibroblasts for 2 d, the dish was filled with 5 mL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum to cover the cover-slide, and the fibroblasts were incubated for a further 8 d. At the end of the culture period, the cells were very gently rinsed three times in phosphate-buffered saline. Then, 3 mL of methanol at −10°C was added, for 10 min, to fix the cells. After the cells had been washed twice in phosphate-buffered saline, 200 μL of a substrate solution for alkaline phosphatase (Nitro Blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate dipotassium) (Zymed, South San Francisco, CA, USA) was added to the cover slides, and the cells were incubated for 30 min at room temperature. The cells were then washed twice in phosphate-buffered saline, and 200 μL of Neutral Red was applied as a counter-stain. The slide was then directly transferred to a light microscope and microphotographed. The quantification of the staining results has been described previously (24,25).

Mineralization activity (Alizarin Red staining)

Ten milligrams of either bone graft was first placed on a sterile glass cover slide (24 × 24 mm) in the middle of a 3-cm culture dish. Approximately 5×10^4 fibroblasts, in 200 μL of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, were added to the cover slide. After incubation of the fibroblasts for 2 d, the dish was filled with 5 mL of Dulbecco's modified Eagle's medium, containing 10% fetal calf serum, to cover the cover-slide, and incubation was continued for another 18 d. At the end of the culture period, the cells were very gently rinsed three times in phosphate-buffered saline. Then, 3 mL of 80% ethanol at room temperature was added to fix the cells. The cultures were stained in a solution of 1% Alizarin Red S (pH 6.4) for 2 h. The stained cultures were gently washed several times in distilled water, and the excess stain was removed by destaining for 10 min in 95% ethanol containing 5% concentrated HCl. The samples were then air dried and transferred for microphotographs. Because both graft materials consisted of hydroxyapaptite [$Ca_{10}(PO_4)_6(OH)_2$], which is reactive to calcium staining, to exclude false-positives, we calculated the positive areas surrounding the bone grafts, but not those in direct contact with the grafts.

Preparation of cell lysates, collection of bound proteins, and gel electrophoresis

Once the periodontal ligament fibroblast cell culture reached confluence, 1 mL of lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol tetraacetic acid, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4) was added to the culture dish and the cell lysate was collected using a cell scraper and pipette. The protein concentration of the cell lysate was measured using a modified Bradford method (Bio-Rad, Hercules, CA, USA). To gather the proteins bound to the bone grafts, 300 μg of protein from the cell lysate was mixed, in a volume of 250 μL, with 25 mg of either bone graft, which had been pretreated with bovine serum albumin to reduce nonspecific binding. The mixed solution in a microcentrifuge tube was incubated in a rocking platform overnight at 4°C. This complex was washed four times with phosphate-buffered saline, and then 40 μL of sample buffer (160 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 26% glycerol, 0.1% bromophenol blue) was added to each tube. These samples were placed in a boiling water bath for 5 min to elute bound proteins, and then analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 10 μL of molecular weight standard (Fermentas Inc., Hanover, MD, USA), and 20 μL of total cell lysate. The gels were fixed and stained overnight in 0.1% Coomassie blue in 12.5% trichloroacetic acid, and then, 24 h later, destained overnight in 7% acetic acid.

Mass spectrometry

We selected the two most obvious bands of proteins to undergo mass spectrometry. The bands, corresponding to ≈38 and ≈54 kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining, were excised with a sterile scalpel and transferred to two separate 0.5 mL microcentrifuge tubes. The samples were then sent to a proteomics core laboratory in our university for further procedures. Briefly, the gel piece was washed twice with 50% (v/v) acetonitrile and 50% (v/v) acetonitrile/25 mM ammonium bicarbonate. Then, the gel fragments were placed in 25 mM ammonium bicarbonate solution with 10 mM dithiothritol and 55 mM iodoacetamide at 56°C for 45 min to be reduced and alkylated. The gel fragments were then digested overnight by modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate at 37°C. The supernatant

was transferred to a microcentrifuge tube, and the peptides were further extracted from the gel piece by incubating it in 50% acetonitrile/5% formic acid. Twenty milliliters of 5% acetonitrile/0.1% formic acid was added to the sample, which was then subjected to mass spectrometry analysis for protein identification. The protein tryptic digests were fractionated using a reverse-phase nano-high-performance liquid chromatography (HPLC) system (LC Packings; Dionex, Amsterdam, the Netherlands) as follows: column, Agilent C_{18} reverse-phase column (3.5 μm, 150 × 0.075 mm) (Agilent, Palo Alto, CA, USA); solvent, 0.1% formic acid with acetonitrile gradient (5–48% in 40 min); flow rate, 0.2 μL/min. As peptides eluted from the microcapillary column, they were electrosprayed into the electrospray ionization-tandem mass spectrometer using a 1.3-kV spraying voltage. The mass spectrometer (QSTAR Pulsar I™; Applied Biosystems, Foster City, CA, USA) was equipped with an ion trap and a nanoelectrospray interface (nanoESI). Product ion scan data obtained from mass spectrometry experiments were identified using MASCOT search (http://www.matrixscience.com) (Matrix Science Inc., Boston, MA, USA).

Subcellular fractioning and western blotting

The results of the mass spectrometry were further confirmed using western blotting. The localization of the proteins in periodontal ligament cells were checked using subcellular fractioning, as previously described (26). The total number of cellular proteins were divided into cytosolic, non-nuclear membrane, and nuclear fractions. Ten microliters of molecular weight standards, and 40 μg of proteins from different fractions, were loaded onto a 12% polyacrylamide gel and separated using electrophoresis. Then, the proteins were electroblotted to poly(vinylidene difluoride) membranes. After they had been blocked in 10% skimmed milk for 1 h at 37°C, the membranes were incubated for 2 h at 37°C with primary antibodies (0.1 μg/mL of polyclonal rabbit antihuman annexin II; H-50, #sc-9061; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal mouse antihuman vimentin (Sigma-Aldrich, St Louis, MO, USA). The blots were then washed and incubated with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h at 37°C. The membrane was washed and then developed and observed using a chemiluminescent detection kit (ECL®; Amersham Pharmacia Biotech, San Francisco, CA, USA) and X-ray films.

Neutralizing experiments

In order to clarify the role of annexin II on the *in vitro* functions of a P-15 xenograft, different concentrations (0–200 μg/mL) of antibody to annexin II (H-50, #sc-9061; Santa Cruz Biotechnology) were added to the resuspended periodontal ligament fibroblasts ($\approx 2 \times 10^4$ fibroblasts in 500 μL of Dulbecco's modified Eagle's medium) and incubated on a rocking platform for 30 min at 37°C. Then, the treated cells were cocultured with 10 mg of P-15 bone graft for alkaline phosphatase activity and MTT assays, as previously mentioned. Ma *et al.* (27). proved that the antibody blocked binding between annexin II and its ligand. A nonspecific rabbit IgG was used as the negative control (Jackson ImmunoResearch Laboratories, Philadelphia, PA, USA). The resulting data were normalized with the average value obtained from the cell cultures co-incubated with anorganic bovine mineral (N-300) alone.

Statistical analysis

The quantification of MTT and alkaline phosphatase activity data are presented as mean ratio ± standard deviation. The results of alkaline phosphatase and mineralization staining are expressed as the percentage of positive cells or area ± standard deviation. One-way analysis of variance, with Fisher's protected least significance as the posthoc test, was used to determine whether significant differences existed between bone graft groups and control groups. Data from six cell-culture studies are representative of at least two independently performed experiments. Statistical significance was set at $p < 0.05$. The statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA, USA) on a PC-compatible computer.

Results

In vitro osteogenesis-associated assays

The MTT value from the cell culture co-incubated with P-15 was significantly greater than that of the cell culture co-incubated with N-300 (anorganic bovine mineral) alone. The cell culture without bone graft had a significantly smaller MTT value than the other two groups (Fig. 1). After it had been adjusted using relative cell numbers (MTT values), the alkaline phosphatase activity from the total cell lysate of the P-15 group was significantly higher than that of the N-300 and negative-control groups (Fig. 2). There was no significant difference between the N-300 and negative-control groups. The positive reaction of

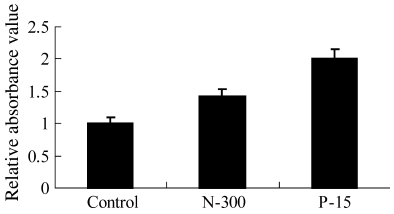

Fig. 1. Cell viability assay (MTT assay) for periodontal ligament fibroblasts co-incubated with either P-15-coated anorganic bovine mineral, or anorganic bovine mineral alone, for 3 d. P-15 denotes the anorganic bovine mineral coated with P-15 peptide; N-300 was the anorganic bovine mineral with the same particle size as P-15 bone graft (range, 250–420 μm; average, 300 μm). Relative absorbance values are means ± standard deviation ($n = 6$). The data are representative of two separate experiments. There were significant differences ($p < 0.001$) between the P-15 and N-300 groups as well as between the N-300 and control groups. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide.

the cytochemical staining for alkaline phosphatase activity appeared to be dark purple (Fig. 3; green arrows). The average percentages of positive cells in the three groups were 8.20 ± 0.64 (negative control), 8.40 ± 0.88 (N-300), and 19.60 ± 2.32 (P-15). The P-15 group had significantly more positive cells than the other two groups. Many positive cells in the P-15 group seemed to be in direct contact with the graft material (Fig. 3). In the Alizarin Red S staining for mineralization, no positive stain was detected in the negative control group (data not shown). Both of the graft materials and every particle of the grafts yielded a positive reaction (Fig. 4). There may have been some overlap between the reaction of graft materials and cells in contact with the grafts. In order to exclude false-positive areas, we measured only the positive areas away from grafts. The average percentages of positive areas were 1.56 ± 0.51 for the N-300 group and 5.14 ± 0.73 for the P-15 group, a significant difference (Fig. 4).

Proteins bound on the grafts detected using mass spectrometry

Coomassie blue staining showed many visible bands with different densities between the N-300 and P-15 groups (Fig. 5). Two of the most apparent bands appeared to be ≈38 and 54 kDa, which corresponded to the molecular weight markers. The bands at ≈54 kDa seemed not to be different among the N-300, P-15, and cell lysate control groups. The bands at ≈38 kDa were more intense in the P-15 group than in the N-300 and control groups. The proteins at ≈54 and ≈38 kDa were extracted and trypsinized to yield different peptide fragments. After analysis by reverse-phase nano-HPLC electrospray ionization/mass spectrometry, 300 queries of peptide fragmentation patterns for each protein were searched in the Mascot database. Probability-based Mowse scores were then calculated. Individual scores of > 44 indicated identity or extensive homology ($p < 0.05$). The search results suggested that human vimentin and annexin II were the most probable proteins for the bands at ≈54 kDa and ≈38 kDa, respectively. The individual ion scores were 1,900 for vimentin and 1,789 for annexin II. Vimentin and annexin II were matched by 79 and 55 unique peptide sequences, respectively. The nominal mass of the human vimentin is 53,653, and the calculated pI value is 5.06. The nominal mass of human annexin II is 38,580, and the pI value is 7.57.

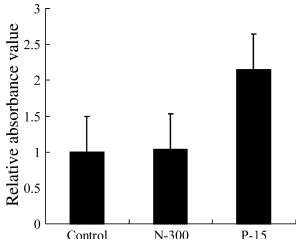

Fig. 2. The comparison of alkaline phosphatase activity from total cell lysates of periodontal ligament cells and those co-incubated with N-300 or P-15. Relative absorbance values are mean ± standard deviation ($n = 6$) The data are representative of two separate experiments. The value of P-15 is significantly greater than those of the N-300 and control groups ($p = 0.03$ and 0.04, respectively).

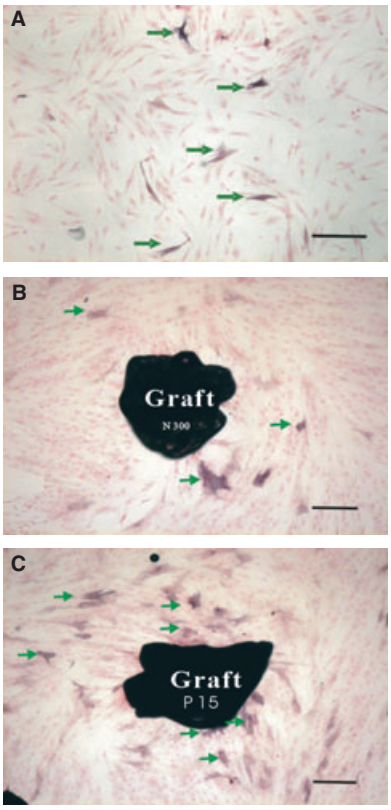

Fig. 3. Cytochemical analysis of alkaline phosphatase activity of periodontal ligament cells cultured on glass slides. The alkaline phosphatase activity was detected with Nitro Blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate dipotassium, a substrate for alkaline phosphatase. The positive reaction appears as dark purple (green arrows). Neutral Red was used for counterstaining. Many cells that reacted positively are in direct contact with the P-15 bone graft (original magnification, × 50; scale bar = 150 μm).

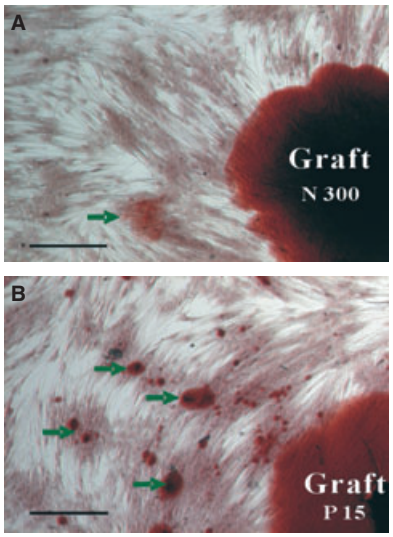

Fig. 4. Mineralization assay (Alizarin Red S assay) for periodontal ligament cells co-incubated with N-300 (A) or P-15 (B) graft materials for 20 d. Both of the graft materials consisted of hydroxyapatite, which causes a positive reaction. We did not calculate the positive area directly surrounding the graft materials. Green arrows indicate the area with positive reaction (original magnification, × 100; scale bar = 150 μm).

Western blotting and subcellular localization

The results of western blotting further confirmed the results of Coomassie blue staining and mass spectrometry. There were two visible bands on the lane of cell lysate from periodontal ligament fibroblasts for the immunoblot of vimentin. The band with a smaller molecular weight was very faint on the P-15 and N-300 lanes. In order to exclude the possibility that the detected proteins were from the P-15

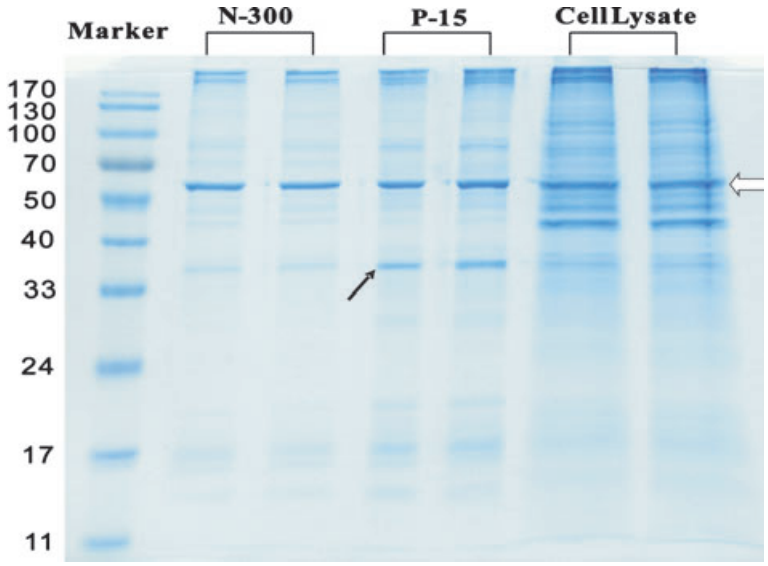

Fig. 5. Coomassie blue staining for bound proteins eluted from bone grafts co-incubated with periodontal ligament cell lysates. Two lanes in each group represent duplicated data. The cell lysate from periodontal ligament fibroblasts was a positive control. The black arrow indicates that the protein at ≈38 kDa (molecular weight) is clearly more abundant in the P-15 group. The white arrow indicates that the protein at ≈60 kDa is equally abundant in all three groups. These two bands of proteins were excised and sent for mass spectrometry analysis.

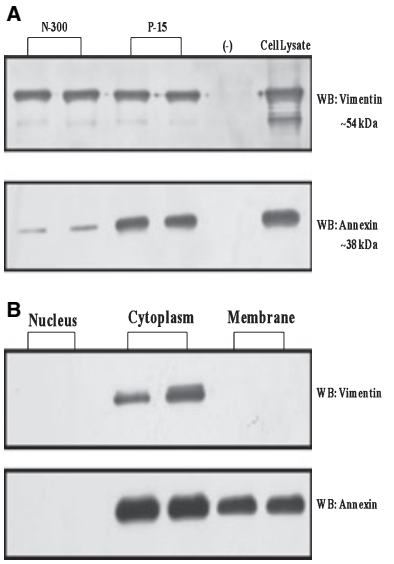

Fig. 6. Western blotting and subcellular localizations of the bound proteins from bone grafts. (A) Western blotting to confirm the bound proteins from N-300 and P-15. Two lanes in each group represent duplicated data. The negative (–) control lane was the eluted solution from washed P-15 graft co-incubated with bovine serum albumin and underwent the same immunoblotting procedure. Cell lysate from periodontal ligament fibroblasts was the positive control. (B) Subcellular localizations of vimentin and annexin II. Fractioning of the subcellular components of periodontal ligament fibroblasts was performed using an ultracentrifugation method (26). The fractioned proteins were then analyzed using the same western blotting analysis used for vimentin and annexin II. WB, western blot.

graft material instead of bound periodontal ligament proteins, the same amount of bovine serum albumin as periodontal ligament proteins was mixed with the P-15 material as the negative control. No visible band was noted on the negative control lane. The annexin II immunoblotting results revealed that significantly more protein was bound to P-15 than to N-300. The negative control lane was clear (Fig. 6A). The results of western blotting for different cellular components demonstrated that vimentin existed only in the cytoplasm, whereas annexin II existed in both the cell membrane and cytoplasm (Fig. 6B).

Neutralizing experiments

The results of MTT and alkaline phosphatase activity assays, which included neutralizing antibody against annexin II, showed that the neutralizing antibody dose-dependently reduced the MTT and alkaline phosphatase activity values of periodontal ligament fibroblast cocultured with P-15 (Fig. 7A,B). There were no significant differences between the groups treated with various concentrations of control rabbit immunoglobulin G. Adding 200 μg/mL of neutralizing antibody decreased alkaline phosphatase and MTT values from the cells without neutralizing antibody to ≈36.2% and 19.3%, respectively.

Discussion

The results of our *in vitro* osteogenesis assays were in agreement with most other studies (2,11,13,14). Bhatnagar *et al.* (11,13,14) reported that more periodontal ligament or dermal fibroblasts were associated with P-15 bone grafts than with grafts containing anorganic bovine mineral alone. Increased mineralization detected using Alizarin Red staining was found on the P-15 group. Kübler *et al.* (2) investigated the effect of different bone graft substitutes, including anorganic bovine mineral and P-15, on the growth and proliferation pattern of human bone cells. P-15 showed the highest proliferation and differentiation rate; anorganic bovine mineral and other bone substitutes showed lower proliferation and differentiation rates. Many *in vivo* human studies (9,15–17,28) also demonstrated that P-15 graft provided better bone healing than anorganic bovine mineral alone in periodontal osseous lesions or maxillary sinus cavities. Although the peptide-coated graft has increased biomineralization *in vitro* and *in vivo*, little information about its molecular mechanism is available. The cell-binding peptide from type I collagen (P-15) was first introduced in 1996 (11). The peptide is in the sequence 766GTPGPQGIAGQRGVV780 of the α1 chain of type I collagen. It is very potent in competition with the natural form of type I collagen for fibroblast cell binding. The strong binding ability of this peptide to fibroblasts may be a

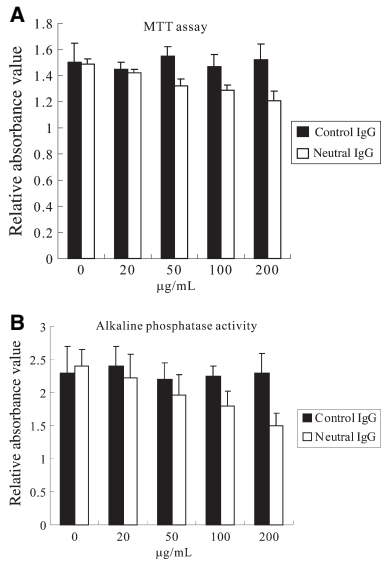

Fig. 7. Neutralizing assays to determine the functions of annexin II in the *in vitro* activities of the P-15 bone graft. (A) MTT assay for periodontal ligament cells co-incubated with P-15 and different concentrations of neutralizing antibody against annexin II or control antibody. The absorbance values from each group were normalized with the absorbance value obtained from the anorganic bovine mineral (N-300) group by adding 200 μg/mL of control antibody. Relative absorbance values are means ± standard deviation ($n = 6$). The data are representative of two separate experiments. (B) Alkaline phosphatase activity assay for periodontal ligament cells co-incubated with P-15 and different concentrations of neutralizing antibody against annexin II or control antibody. The absorbance values from each group were normalized with the absorbance value obtained from the anorganic bovine mineral (N-300) group by adding 200 μg/mL of control antibody. Relative absorbance values are means ± standard deviation ($n = 6$). The data are representative of two separate experiments. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide.

result of the local conformation change between the collagen fold and beta-bend (12). However, it is still unclear why the strong binding effect can increase the differentiation of mineralization-associated cells (e.g. periodontal ligament fibroblasts and osteoblasts). Several studies (13,14,18, 19,29) pointed out that P-15 peptide not only up-regulated the expression of transforming growth factor-β1, but also down-regulated one transforming growth factor-β1 inhibitor. Transforming growth factor-β1 is thought to be important in the development, induction, and repair of bone (30,31). Nonetheless, there is still missing information between the binding of fibroblasts to P-15 bone grafts and the production of downstream effects, such as up-regulating transforming growth factor-β1.

Mass spectrometry allowed us to identify two proteins abundantly bound to P-15 graft material. Vimentin is an intermediate filament and is typical of cells of mesenchymal origin (32). In oral tissues, it has been immunolocalized in fibroblasts of the periodontal ligament and dental pulp, in odontoblasts, and in fibroblasts of the dental papilla and dental follicle during tooth development (33,34). Vimentin is important for vital mechanical and biological functions, such as cell contractility, migration, stiffness, signal transduction, proliferation, and lipid droplet accumulation (35). However, vimentin knockout mice demonstrate very subtle phenotypes not obviously related to cell fragility (36). Our findings suggested that vimentin is localized only in the cytoplasm of periodontal ligament fibroblasts; there was no significant difference in the amount binding to P-15 and anorganic bovine mineral bone grafts. We did not further explore the role of vimentin in mineralization.

There was significantly more annexin II, the second protein revealed using mass spectrometry, bound to P-15 bone graft than anorganic bovine mineral. Annexins are a family of Ca^{2+}-binding membrane proteins. There are more than 160 unique annexin proteins in more than 65 different species, ranging from fungi and protists to plants and higher vertebrates (37). This family of proteins has been implicated in endo- and exocytosis, membrane fusion, membrane/cytoskeletal interactions, and voltage-dependent calcium channels (38). Annexin II has been described as a cell-surface receptor for extracellular matrix molecules, such as tissue plasminogen activator, cathepsin B, and tenascin-C (39–41). Annexin II has not only been detected in the cytoplasm and plasma membrane (42), but is also a major component of extracellular matrix vesicles (43). Matrix vesicles are particles that, after being released from the plasma membrane of hypertrophic chondrocytes or osteoblasts, have the critical function of initiating the mineralization process in cartilage and bone (42–44). It was believed that the annexin II associated with collagen formed a calcium channel that allows the rapid accumulation of Ca^{2+} in the matrix vesicles (44,45). Matrix vesicles, containing relatively high concentrations of Ca^{2+} and inorganic phosphate, create an optimal environment to induce the formation of hydroxyapatite. Furthermore, annexin II and alkaline phosphatase (an important enzyme in the mineralization process) were found to be localized in membrane microdomains called 'lipid rafts' in osteoblasts (42). Annexin II overexpression increased alkaline phosphatase activity and mineralization in both chondrocytes and osteoblasts. Both the disruption of lipid rafts and reduced expression of annexin II diminished mineralization (42,46). That is, annexin II on both extracellular matrices and cell membranes are important for mineralization.

We found that P-15 increased periodontal ligament mineralization *in vitro*, and that the addition of 200 μg/mL of neutralizing antibody against annexin II decreased by approximately 36.2% of the alkaline phosphatase activity of cells cocultured with P-15 bone graft. This suggested that annexin II was partly responsible for the *in vitro* osteogenesis effect of P15. Several studies (47–50) have reported that type I collagen enhances alkaline phosphatase activity and mineralization. From the limitations of our data, it is still unclear whether P-15 peptide is the only structural domain responsible for the role of type I collagen in mineralization. The development of a specific blocking antibody for this 15-amino-acid sequence of type I collagen will help to answer the question. Although our results demonstrated that annexin II significantly

bound to P-15, it is still unclear whether the binding per se activated the calcium channel and led to mineralization or increased mineralization using indirect pathways. Studies designed to measure the Ca^{2+} currents, influx, or intracellular Ca^{2+} signaling of periodontal ligament cells after they bind to P-15 bone graft are needed to answer this question. In summary, the present study demonstrated that P-15 bone graft increased the *in vitro* osteogenesis activities of periodontal ligament fibroblasts. A mineralization-associated membrane protein, annexin II, significantly bound to the P-15 graft material and was partially responsible for the *in vitro* osteogenic effects of P-15.

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