ARTICLE
Journal of Cellular Biochemistry 113:1724–1732 (2012)

Journal of Cellular Biochemistry

Streptococcus Mutans GS-5 Antigen I/II Stimulates Cell Survival in Serum Deprived-Cultures Through PI3K/Akt Pathways

Young-Ok Son,[1] Young-Mi Jeon,[2] Yu-Shin Kim,[2] Soon-Sun Park,[2] Seung-Moon Park,[3] Ji-Hae Kim,[2] and Jeong-Chae Lee[1,2*]

[1] *Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky 40536-0001*

[2] *Institute of Oral Biosciences and BK21 Program, School of Dentistry, Chonbuk National University, Jeonju 561-756, South Korea*

[3] *Division of Biotechnology, Chonbuk National University, Iksan 570-752, South Korea*

ABSTRACT

The antigen I/II (AgI/II) protein is a major surface protein that mediates the attachment of *Streptococcus mutans* (*S. mutans*) to the saliva-coated pellicle. Numerous studies have investigated not only the mechanisms by which AgI/II signaling is transduced within cells, but have also attempted to use AgI/II-specific antibodies to treat dental caries and host immune responses. However, little information is available about the effects of AgI/II on basic cellular events in bone cells. In this study, we examined the effects of the His-tagged recombinant N-terminal half of the AgI/II protein (rAgI/II-N) generated from *S. mutans* GS-5 on the viability, proliferation, and cell cycle progression of primary calvarial osteoblasts. We also investigated the mechanisms involved in the rAgI/II-N-mediated survival of serum-starved osteoblasts. We found that rAgI/II treatment attenuated the serum deprivation-induced decrease in cell viability and proliferation of osteoblasts. rAgI/II-N also prevented the loss of mitochondrial membrane potential (MMP), alterations in levels of two key mitochondrial Bcl-2 family proteins, and the accumulation of numerous cells into the sub-G_1 phase that were observed in serum-starved osteoblasts. Pharmacological inhibitors of phosphoinositide 3-kinase (PI3K), but not of extracellular signal-regulated kinase or Ras, blocked the rAgI/II-N-mediated protection against serum deprivation-induced cell death. Additional experiments revealed that the integrin α5β1-mediated PI3K pathway is required for rAgI/II-N-mediated Akt phosphorylation in osteoblasts. Collectively, these results suggest that rAgI/II-N induces survival signals in serum-starved osteoblasts through integrin-induced PI3K/Akt signaling pathways. J. Cell. Biochem. 113: 1724–1732, 2012.

KEY WORDS: RECOMBINANT AgI/II; CALVARIAL OSTEOBLASTS; SURVIVAL; CELL CYCLE PROGRESSION; SIGNAL TRANSDUCTION PATHWAYS

Streptococcus mutans (*S. mutans*) is known as the most important mediator of dental caries [Loesche, 1986]. Many investigators have focused their efforts on developing vaccines or passive immunization protocols that are capable of preventing *S. mutans*-mediated diseases [Kitten et al., 2002; Han et al., 2006]. The results of numerous studies suggest that vaccination using peptides derived from a surface protein of *S. mutans*, antigen I/II (AgI/II), is a very promising strategy for controlling and preventing dental caries [Robinette et al., 2011].

Because the role of AgI/II in host responses is important, many studies have explored the mechanisms by which the AgI/II signal is transduced within cells. AgI/II is known to bind directly to dentinal cell surface receptors such as α5β1 integrins and Toll-like receptor 4 [Hajishengallis et al., 2002]. This binding induces the expression of surface molecules required for adhesion and migration of cells [Heddle et al., 2003; Nobbs et al., 2007]. Especially, integrins are known to play important roles in transducing extracellular stimulation into cellular signals and thereby affecting cellular events such as proliferation, differentiation, and apoptosis. Li et al. [2011] demonstrated the contribution of integrin-mediated signaling in the promotion of cell survival and cell cycle progression. It was also reported that the activation of integrin-linked kinase

Young-Ok Son and Young-Mi Jeon contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Prof. Jeong-Chae Lee, Institute of Oral Biosciences (BK21 program) and School of Dentistry, Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, South Korea.
E-mail: leejc88@jbnu.ac.kr

Received 21 September 2011; Accepted 21 December 2011 • DOI 10.1002/jcb.24042 • © 2011 Wiley Periodicals, Inc.
Published online 28 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

decreased apoptosis in serum deprived-endothelial progenitor cells [Zhao et al., 2011]. Further, integrin signaling is capable of regulating the switch between tumor suppression and promotion through the interaction with extracellular matrix [Keely, 2011]. These reports suggest that AgI/II affects a variety of cellular responses by binding to integrins and/or activating their expression in host cells.

The binding of AgI/II to cell surface molecules also leads to the synthesis and release of pro-inflammatory cytokines in periodontal tissues. Actually, AgI/II stimulated the release of tumor necrosis factor and interleukins in periodontal cells [Engels-Deutsch et al., 2003]. *S. mutans* AgI/II bound to α5β1 integrins and also increased the expression of β1 integrin in periodontal ligament cells [Engels-Deutsch et al., 2011]. It is important to note that a chronic interaction between periodontal fibroblasts and oral pathogenic bacteria causes degradation of periodontal collagens, forms pockets around teeth, and eventually results in tooth loss [Ara et al., 2009]. Moreover, periodontal-pathogenic bacteria can bind directly to alveolar bone in late periodontitis and disrupt a balanced activation of osteoblasts and osteoclasts which are required for bone remodeling. However, little is known about the effect of AgI/II binding on basic cellular events in bone cells. The mechanisms by which AgI/II affects cell proliferation and survival of osteoblasts remain unclear.

In this study, we examined the effects of AgI/II on the viability, proliferation, and cell cycle progression of primary calvarial osteoblasts using the His-tagged recombinant N-terminal half of the AgI/II protein (rAgI/II-N). We also investigated the mechanisms involved in the rAgI/II-N-mediated survival of osteoblasts under serum starvation conditions. In addition, we compared the effect of rAgI/II-N on mouse primary osteoblasts to that of MC3T3-E1 preosteoblastic cells or NIN3T3 fibroblastic cells.

MATERIALS AND METHODS

MATERIALS

PD98059, an extracellular signal-regulated kinase (ERK) inhibitor, was purchased from Tocris Bioscience (Minneapolis, MN). Wortmannin and Ly294002, inhibitors specific for phosphoinositide 3-kinase (PI3K), were obtained from Calbiochem (San Diego, CA). Ras inhibitor and fetal bovine serum (FBS) were purchased from Enzo Life Science (Ann Arbor, MI) and Hyclone (Logan, UT), respectively. Unless otherwise specified, other chemicals, antibodies, and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO), Santa Cruz Biotechnology (Santa Cruz, CA), and SPL Life Sciences (Pochun, South Korea), respectively. rAgI/II-N, which was produced from *S. mutans* GS-5 using the pQE vector containing *agI/II*-N gene [Jeong et al., 2005; Jeon, 2006], because of the unique feature of the stain; it lacks part of the C domain and the cell wall anchoring motif with a molecular weight of 155 kDa [Murakami et al., 1997; Jeon, 2006] and also produces a secreted and truncated AgI/II protein that has been utilized as a vaccine candidate [Nakano et al., 2006]. The rAgI/II-N was a kind gift from Prof. K.-Y. Lee (Department of Oral Microbiology, Chonbuk National University). The rAgI/II-N used in this study was obtained from the soluble fraction of bacterial lysates after application to a Ni-NTA column and was more than 90% pure [Jeon, 2006].

CELL CULTURE

Animal care and use were approved by the Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals. Primary osteoblasts were prepared from the calvariae of 7-day-old BALB/c mice by repeated digestion of the calvariae with 0.05% trypsin and 0.1% collagenase at 37°C. Primary osteoblasts were cultured in α-minimum essential medium (α-MEM) supplemented with 10% FBS and antibiotics. Murine osteoblastic MC3T3-E1 cells (ATCC, CRL-2593) were cultured in the same medium and were used as a counterpart to the primary osteoblasts. NIH3T3 cells (ATCC, CRL-1658) were also cultured in Dulecco's modified Eagle medium containing 10% bovine calf serum and used for the analysis of proliferation according to the presence and absence of rAgI/II-N or serum. For the serum starvation experiments, cells were harvested in the exponential growth phase and plated at a density of 1×10^6 cells/ml in 6-well plates or 96-multiwell plates in serum-free medium containing various concentrations of rAgI/II-N and/or each of the pharmacological inhibitors. At various incubation times, cells were processed to analyze viability, proliferation, cytotoxicity, cell cycle progression, and protein expression.

MEASUREMENT OF CELL VIABILITY AND CYTOTOXICITY

We used 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) assays to evaluate the viability of cells. At various incubation times, 10 μl of MTT solution (5 mg/ml in PBS as stock solution) was added into each well of 96-well plates, followed by a 4-h incubation at 37°C. The reduction of MTT due to cellular activity was measured by treating the cells with acidic isopropanol prior to reading the absorbance of the dissolved formazan crystals at 560 nm using a SpectraCount™ ELISA reader (Packard Instrument Co., Downers Grove, IL). In addition, cytotoxicity was determined by staining aliquots of cells with 0.4% trypan blue; approximately 100 cells were counted for each treatment. Cell death was calculated as follows: % cytotoxicity = (total cells − viable cells)/total cells) × 100%.

MEASUREMENT OF DNA SYNTHESIS

The level of DNA synthesis by cells was measured by adding 10 μCi of ^{3}H-thymidine deoxyribose (TdR, 10 μCi; Amersham Pharmacia Biotech Inc., Piscataway, NJ) to each well of 96-multiwell plates during the last 12 h prior to cell harvesting. Cells were collected from the plates using a trypsin-EDTA solution and resuspended in PBS before aliquoting them into 96-multiwell plates. Cells were subsequently collected using a harvester (Inotech Inc., Dietikon, Switzerland), and beta emission from the ^{3}H-TdR-incorporated cells was measured for 1 min using a liquid scintillation counter (Packard Instrument Co.).

CELL CYCLE ANALYSIS

Cells were harvested and washed briefly with ice-cold PBS. The cells were then fixed in cold 70% ethanol and stored for 2 h at −20°C prior to staining. Fixed cells were washed with PBS and resuspended in a solution containing 1.12% sodium citrate (pH 7.4), DNase-free

RNase I (500 U/ml), and propidium iodide (PI, 50 μg/ml) for 1 h at room temperature in the dark. After staining, the PI intensity in cells (1×10^4 cells) was analyzed using a FACS Calibur® system (Becton Dickinson, San Jose, CA) and cell cycle progression was determined using WinMDI software (version 2.9).

MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL (MMP)

Cells (2×10^6 cells/ml) were resuspended in PBS and stained with 50 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR) for 20 min at 37°C. The fluorescence related to MMP was measured using a FACS Calibur® system (Becton Dickinson, San Jose, CA), and the changes in MMP levels were determined using the WinMDI 2.9 program.

PREPARATION OF CELL FRACTIONS

Whole cell lysates were made in NP-40 lysis buffer (30 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF and a protease inhibitor mixture containing 1 μg/ml aprotinin and 1 μg/ml leupeptin). Protein content was quantified using the Bradford method (1976). For mitochondrial fractionation, cells were collected, washed with ice-cold PBS, and incubated with 150 μl of buffer A (250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml each of leupeptin, aprotinin, and pepstatin A) on ice for 30 min. The cells were disrupted by 20 passes through a 26-G needle and centrifuged at 750*g* for 10 min. The pellet containing mitochondria was washed briefly with buffer A and then resuspended in the protein lysis buffer described above. After centrifugation at 10,000*g* for 25 min, the supernatant was used as the mitochondrial fraction to analyze the mitochondrial changes of Bcl-2 family proteins by Western blotting.

IMMUNOPRECIPITATION

β1 integrin was subjected to immunoprecipitation. In brief, 10 μl of 200 μg/ml anti-β1 integrin antibody was added to an equivalent volume of protein lysate and kept overnight at 4°C with occasional shaking. Protein G sepharose beads (25 μl) were added to the lysates, and the mixture was incubated for another 2 h. The sepharose beads were collected by centrifugation, and washed three times with TSA buffer (0.01 M Tris and 0.14 M NaCl, pH 8.0) before SDS–PAGE.

WESTERN BLOT ANALYSIS

Samples of extracts containing equal amounts of protein (30 μg/sample) were analyzed by SDS–PAGE (12–15% gels) and blotted onto polyvinyl difluoride membranes. The blots were probed with primary antibodies and incubated with a horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence reagents (Santa Cruz Biotechnology) and exposed to X-ray film (Eastman-Kodak, Rochester, NY).

STATISTICAL ANALYSIS

Unless specified otherwise, all data are expressed as means ± standard deviations (SD) from three or five independent experiments. A one-way analysis of variance (ANOVA) followed by Scheffe's test was used for multiple comparisons. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

rAgI/II-N TREATMENT INCREASES VIABILITY AND PROLIFERATION AND REDUCES CYTOTOXICITY IN SERUM-STARVED OSTEOBLASTS

The addition of rAgI/II-N at concentrations ranging from 1 to 50 μg/ml to primary osteoblasts cultured in medium containing 10% FBS did not affect the viability of the cells at 48 or 72 h after co-incubation (data not shown). When the cells were incubated in serum-free medium for 72 h, addition of rAgI/II-N increased their viability in a dose-dependent manner, compared to untreated cells (Fig. 1A). A time-course experiment confirmed the serum deprivation-induced and time-dependent reduction in viability and its significant attenuation by rAgI/II-N (Fig. 1B). The serum deprivation-mediated reduction of cell viability and its inhibition by rAgI/II-N was also observed in preosteoblastic MC3T3-E1 cells, whereas such effect was not as apparent as found in primary osteoblasts (data not shown). Trypan blue staining revealed that serum deprivation for 72 h caused 78 ± 3.1% cell death of primary osteoblasts, whereas co-incubation with 5 and 50 μg/ml rAgI/II-N reduced cell death to 55 ± 4.5% and 24 ± 5.1%, respectively (Fig. 1C). In MC3T3-E1 cells,

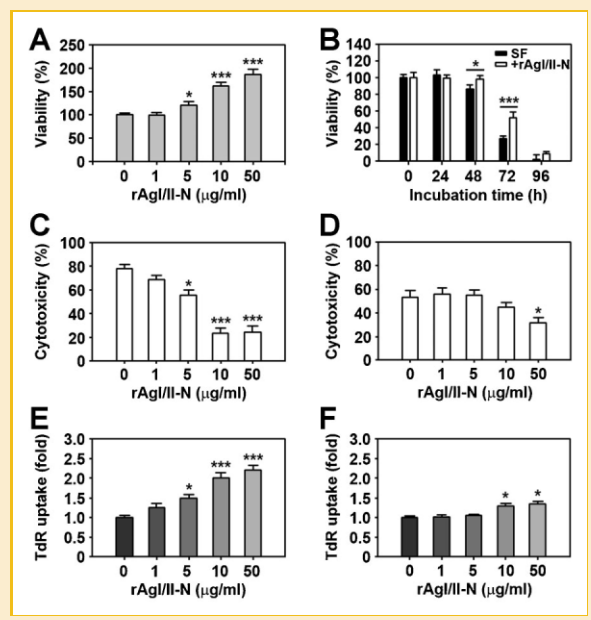

Fig. 1. Effects of rAgI/II-N on the viability and proliferation of serum-starved osteoblasts and determination of its cytotoxicity. Mouse calvarial osteoblasts were incubated in serum-free medium in the presence and absence of various concentrations of rAgI/II-N for 72 h followed by the MTT assay (A), trypan blue staining (C), and TdR incorporation assays (E). In addition, the cells were cultured in serum-free medium containing 50 μg/ml rAgI/II-N and at the indicated times (0–96 h), they were collected and processed for the MTT assay (B). Preosteoblastic MC3T3-E 1 cells were incubated in serum-free medium with the indicated concentrations of rAgI/II-N for 72 h and then the levels of cytotoxicity (D) and TdR uptake (F) were determined. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus rAgI/II-N untreated cells. SF, serum-free.

the significant protection against serum starvation-mediated cytotoxicity was shown when the cells were exposed to 50 μg/ml rAgI/II-N (Fig. 1D). rAgI/II-N treatment increased the incorporation of TdR in osteoblasts in a dose-dependent manner (Fig. 1E). The addition of 5 and 50 μg/ml rAgI/II-N increased TdR uptake levels by 1.5 and 2.2-fold, respectively, compared to untreated cells. However, there was no dose-dependent increase in DNA synthesis by the cells when more than 50 μg/ml rAgI/II-N was added to the cultures (data not shown). In MC3T3-E1 cells, 10 μg/ml rAgI/II treatment led to a mild increase in TdR uptake (Fig. 1F). rAgI/II-N-mediated increase in the TdR uptake was further apparent in NIH3T3 cells, where rAgI/II-N treatment at 50 μg/ml increased the TdR incorporation levels by 5.6-fold, compared to untreated cells (Supplement Fig. 1).

rAgI/II-N REDUCES MITOCHONDRIAL STRESS IN SERUM STARVED OSTEOBLASTS

To determine whether rAgI/II-N prevented serum starvation-induced mitochondrial stress, osteoblasts were incubated for 72 h in serum-free medium supplemented with various concentrations of rAgI/II-N and then stained with PI or $DiOC_6$. Quantitative analysis after flow cytometric analysis revealed that rAgI/II-N not only suppressed the migration of cells into the sub-G_1 phase of cell cycle progression (Fig. 2A), but also prevented the loss of MMP in the cells (Fig. 2B). We next evaluated the mitochondrial levels of the Bcl-2 family proteins, Bcl-2 and Bax, which play a role in the immediate mitochondrial apoptotic response. After 48 h of serum starvation, Bcl-2 levels in the mitochondria had almost completely disappeared, with an attendant increase in levels of Bax (Fig. 2C). Co-incubation with 50 μg/ml rAgI/II-N prevented these changes in the levels of the two Bcl-2 family proteins. Quantitative analysis of three independent experiments supported the inhibitory effects of rAgI/II-N on serum starvation-mediated alterations in apoptosis-inhibiting or promoting proteins (Fig. 2D).

rAgI/II-N PROMOTES CELL CYCLE PROGRESSION IN SERUM-STARVED PRIMARY OSTEOBLASTS

To understand the rAgI/II-N-mediated increase in TdR uptake by primary osteoblasts, cell cycle progression was analyzed by flow cytometry after staining cells with PI after 72 h of serum starvation. As the results, rAgI/II-N treatment led to the accumulation of the cell population in the sub-G_1 phase with an attendant decrease in the number of cells in both the G_0/G_1 and G_2/M phases (Fig. 3A). The addition of rAgI/II-N dose-dependently attenuated the number of sub-G_1 cells that were apoptotic. When 5 or 50 μg/ml rAgI/II-N was added to primary osteoblasts, the percentage of cells in the G_2M phase increased to 17 and 39%, respectively, compared to untreated cells (6%) or 10% FBS-treated control cells (52%) (Fig. 3B). This was similar to the results observed when MC3T3-E1 cells were exposed to rAgI/II-N using the same media conditions (Fig. 3C). Western blot analysis shows that rAgI/II-N treatment increased the levels of PCNA, cyclin D1, and cyclin E proteins (Fig. 4A). However, the levels of cyclin-dependent kinase-4 (CDK4) were not affected by the presence of rAgI/II-N protein in serum-starved osteoblasts. rAgI/II-N also decreased the levels of p21 and p27 proteins that were increased in osteoblasts cultured in serum-free medium (Fig. 4B). Quantitative analysis revealed that rAgI/II-N decreased p27 levels to a greater extent than p21 levels (Fig. 4C).

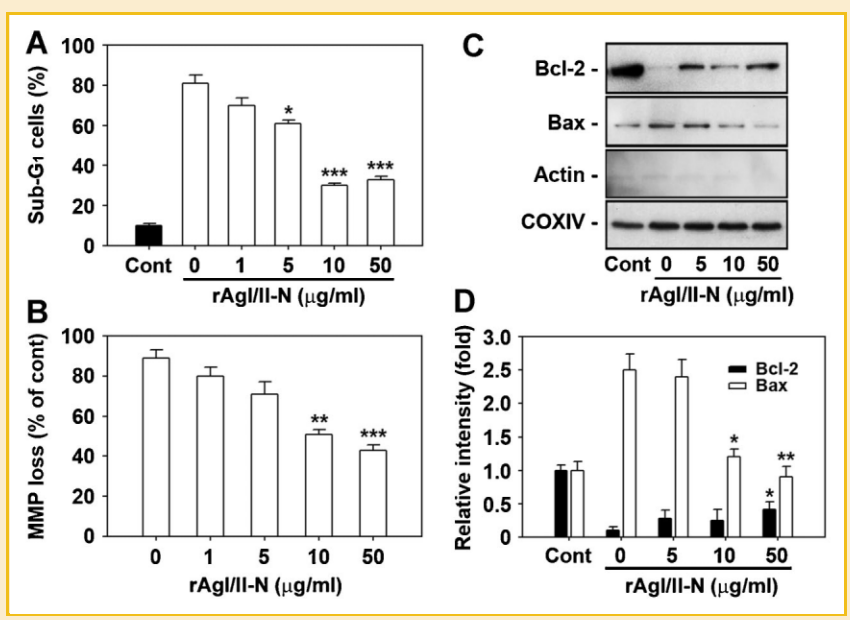

Fig. 2. Effects of rAgI/II-N on the mitochondrial stress caused by serum starvation in calvarial osteoblasts. Osteoblasts were incubated in medium with and without 10% FBS containing the indicated concentrations (0–50 μg/ml) of rAgI/II-N. After a 72-h incubation, the cells were processed for flow cytometric analysis after staining with PI (A) or $DiOC_6$ (B). C: Mitochondrial fractions were prepared from the cells incubated with rAgI/II-N (0–50 μg/ml) for 48 h and then analyzed by Western blotting. D: Protein levels from three independent experiments were analyzed using densitometric analysis after normalizing the intensity of the protein bands to that of cytochrome c oxidase (COXIV). 'Cont' in experiment 'A' indicates cells incubated in the presence of 10% FBS. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus rAgI/II-N-untreated cells.

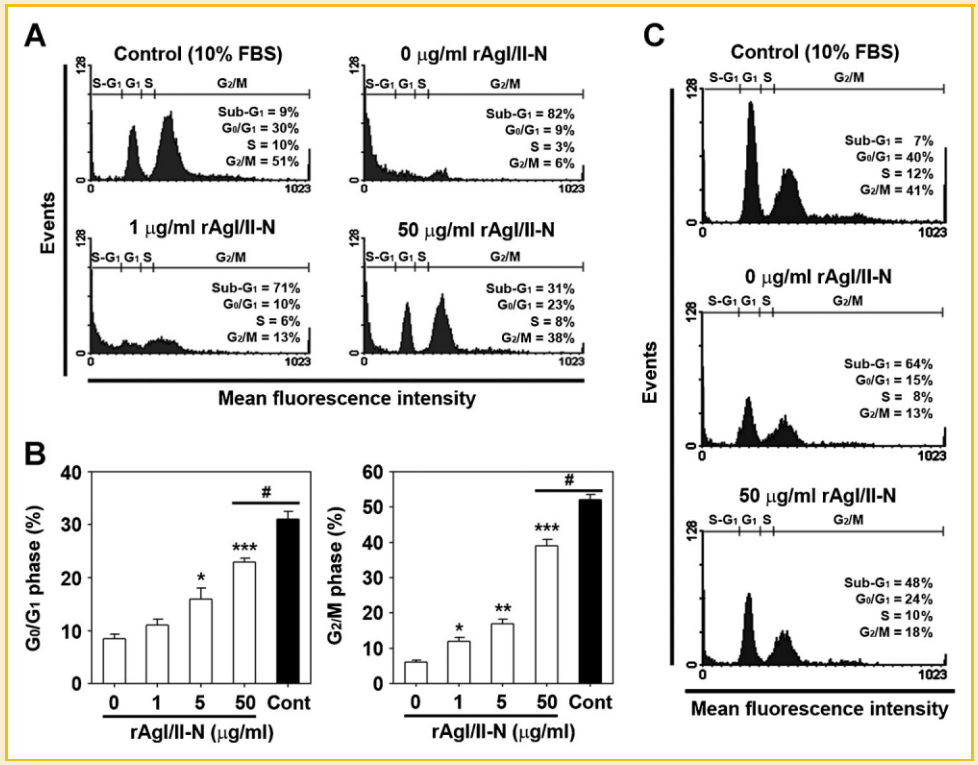

Fig. 3. Promotion of cell cycle progression in serum-starved osteoblasts by rAgI/II-N. A: Calvarial osteoblasts were incubated in medium with or without 10% FBS containing the indicated concentrations (0–50 μg/ml) of rAgI/II-N. After a 72-h incubation, cell cycle progression was analyzed using a flow cytometer after PI staining. B: The percentages in the G_0/G_1 and G_2/M phases were calculated from three independent experiments using the WinDMI 2.9 program. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus rAgI/II-N-untreated cells. $^{\#}P < 0.05$ versus control cells cultured in the presence of 10% FBS. C: MC3T3-E1 cells were also incubated in the same media described above for 72 h and processed for PI staining. A representative result from triplicate experiments is shown.

THE PI3K/AKT SIGNALING PATHWAY IS INVOLVED IN rAgI/II-N-MEDIATED PROTECTION FROM SERUM STARVATION-INDUCED CELL DAMAGE

As the activation of several cellular kinases is required for proliferation, we investigated the effects of rAgI/II-N on the induction of ERK, Akt, and Ras. When osteoblasts were incubated in serum-free medium, ERK phosphorylation was significantly reduced as compared to serum-containing controls, and this was not affected by the presence of rAgI/II-N (Fig. 5A,B). In parallel with this result, rAgI/II-N did not alter the serum deprivation-induced decrease in Ras protein levels. However, rAgI/II-N at 5 or 50 μg/ml treatment blocked the serum deprivation-mediated decrease in Akt phosphorylation. To verify the effects of these kinases on viability, osteoblasts were incubated for 72 h in the presence and absence of various concentrations of pharmacological inhibitors specific for the kinases. rAgI/II-N-mediated increase in cell viability was not affected by treating cells with ERK or Ras inhibitor (data not shown). However, treatment of cells with PI3K inhibitor (Ly294002) reduced the rAgI/II-N-mediated increase in viability in a dose-dependent manner (Fig. 5C). The effects of rAgI/II-N on the accumulation of the sub-G_1 phase (Fig. 6A) and the reduction of MMP levels (Fig. 6B) induced in serum starved-osteoblasts were almost completely disappeared in combined treatment with 10 μM Ly294002.

THE INTEGRIN-MEDIATED PI3K PATHWAY ACTS AS AN UPSTREAM EFFECTOR OF AKT PHOSPHORYLATION INDUCED BY rAgI/II-N IN SERUM-STARVED OSTEOBLASTS

Next, we examined whether PI3K/Akt-mediated signaling is involved in the rAgI/II-N-mediated attenuation of serum starvation-mediated cell death. In serum-starved primary osteoblasts, addition of rAgI/II-N to the cultures increased total PI3K levels accompanied by an increase in p110α and p110β PI3K levels (Fig. 7A). Treatment of cells with a PI3K inhibitor, wortmannin, decreased the rAgI/II-N-stimulated increase in p-Akt levels in serum-starved cells (Fig. 7B). Addition of 100 nM wortmannin for 72 h resulted in a significant suppression of the rAgI/II-N-mediated increase in cell viability (Fig. 7C). In addition, the inhibitor significantly blocked the rAgI/II-N-stimulated DNA synthesis by the cells (Fig. 7D). Similarly, treatment of primary osteoblasts with GSK690693, a pan-Akt kinase inhibitor, suppressed significantly rAgI/II-N-mediated increase in cell viability and DNA synthesis (data not shown). Furthermore, rAgI/II-N treatment increased the levels of α5 and β1 integrins in serum-starved osteoblasts (Fig. 8A). The results from immunoprecipitation using β1 integrin showed a dose-dependent increase in PI3K according to the addition of rAgI/II-N (Fig. 8B). The rAgI/II-N-mediated induction of β1 integrin was not affected by treating cells with 10 μM Ly294002 or 100 nM

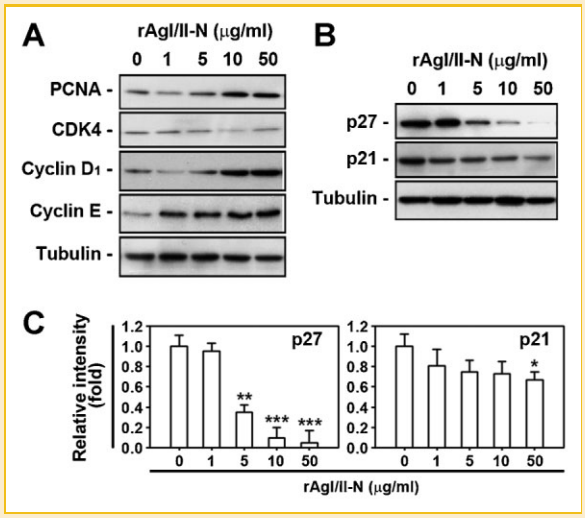

Fig. 4. Regulatory effects of rAgI/II-N on the induction of cell cycle regulatory proteins in serum-starved calvarial osteoblasts. A and B: Cells were exposed to increasing concentrations (0–50 μg/ml) of rAgI/II-N in serum-free medium for 48 h and then processed for Western blot analysis. C: Protein levels from five independent experiments were analyzed using a densitometer after normalizing the intensity of the protein bands to that of tubulin. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus rAgI/II-N-untreated cells.

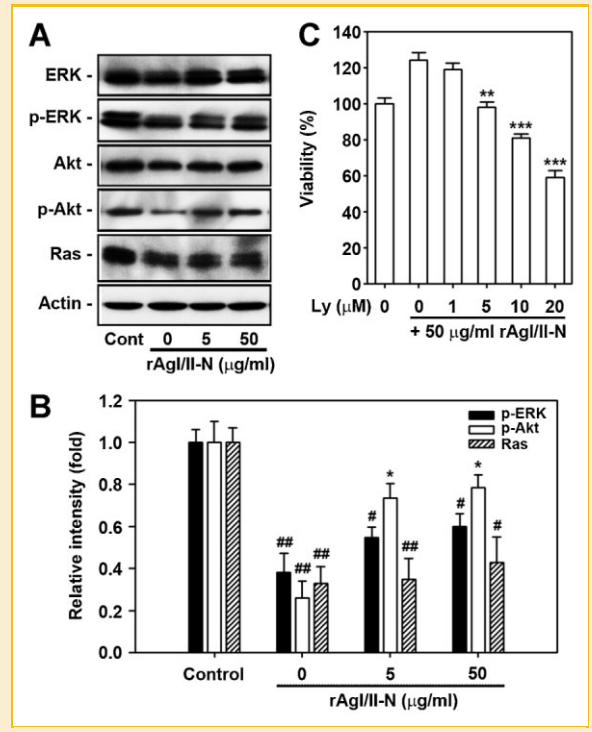

Fig. 5. Effects of rAgI/II-N on levels of protein kinases in serum-starved calvarial osteoblasts. A: Cells were treated with the indicated concentrations of rAgI/II-N in serum-free medium and after 48 h of incubation, protein lysates were prepared followed by immunoblot analysis. B: Protein levels from five independent experiments were analyzed using densitometric analysis after normalizing the intensity of protein bands to that of actin. C: Osteoblasts were also exposed to 50 μg/ml rAgI/II-N in serum-free medium supplemented with various concentrations (0–20 μM) of Ly294002. After a 72-h incubation, the viability of the cells was determined by the MTT assay. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus rAgI/II-N-untreated cells. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus control cells cultured in the presence of 10% FBS. Ly, Ly294002.

wortmannin (Fig. 8C). Similarly, these PI3K inhibitors did not influence rAgI/II-N-mediated increase of α5 integrin in the cells (data not shown).

DISCUSSION

In addition to the implication in dental plaque formation, AgI/II protein has been suggested to play a critical role in host inflammatory response, especially in periodontitis. Reports that an AgI/II-specific monoclonal antibody was capable of modulating host responses after *S. mutans* infection highlights the critical roles of AgI/II in oral and systemic diseases [Crowley et al., 1999; Brady et al., 2000]. There was a close relationship between inflammation and bone loss. A prolonged paradentitis or pulpitis causes degradation of alveolar bone resulting in tooth loss, where the binding of AgI/II to periodontal fibroblastic cells is important event. There was also a report that a significant increase in periodontal bone loss was closely accompanied by elevated expression of pro-inflammatory cytokines and innate immune receptors in old mouse model [Liang et al., 2010]. However, a direct effect of AgI/II on bone cells according to its binding was barely explored.

In the present study, we demonstrate for the first time that AgI/II protein activates survival signaling of osteoblasts in serum-starved cultures. Compared with MC3T3-E1 cells, serum starvation-mediated cytotoxicity and its attenuation by rAgI/II-N were further dramatic in primary osteoblasts. This appeared to be due to a different type of cells, i.e., primary and cell line, and their origins. The present findings also revealed that the effects of rAgI/II-N on proliferation and survival of cells were not limited to bone cells, in that rAgI/II-N strongly increased survival of NIH3T3 cells in serum-deprived cultures. The stimulating effect of rAgI/II-N on proliferation of NIH3T3 cells was shown, even when the cells were cultured in the presence of 10% FBS (data not shown). This supports the stimulating roles of rAgI/II-N on cell survival and proliferation, whereas such effect differs according to the type of cells. It can be also considered that signaling pathways involved in cell survival and proliferation are responded differently to rAgI/II-N and serum, depending on the kinds of cells. Moreover, serum contains a lot of known and unknown growth factors and these factors might stimulate a variety of intracellular signals involved in proliferation and survival. Taken together, the current findings support strongly a protective role of AgI/II protein against serum deprivation-mediated cell death in bone-like cells. More detailed experiments to explain a possible mechanism involved in the different sensitivity of cells to rAgI/II-N will be needed.

Cell survival can be regulated via integrin-mediated control of cell morphology and adhesion in combination with growth factors or intracellular protein kinases linked to integrins. Numerous studies have suggested the involvement of various integrin heterodimers

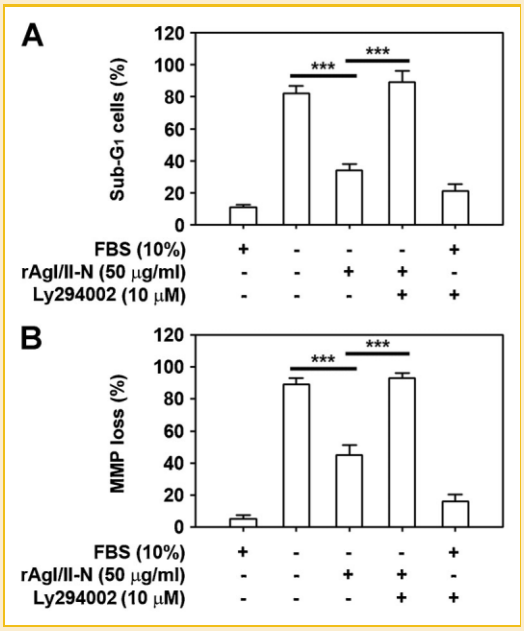

Fig. 6. PI3K specific inhibitor blocks the inhibitory effects of rAgI/II-N on the accumulation of cells in sub-G_1 phase and the loss of MMP caused by serum starvation. Osteoblasts were incubated in the presence or absence of 10% FBS, 50 μg/ml rAgI/II-N, and/or 10 μM Ly294002. After a 72-h incubation, the cells were processed for flow cytometric analysis after staining with PI (A) or $DiOC_6$ (B). $^{***}P < 0.001$ indicates a significant difference between experiments.

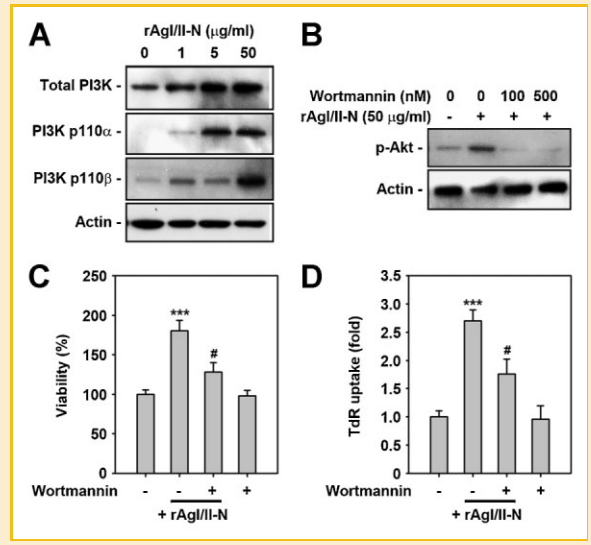

Fig. 7. Involvement of the PI3K pathway in rAgI/II-N-mediated Akt phosphorylation and survival in serum-starved osteoblasts. A: Cells were treated with the indicated concentrations of rAgI/II-N in serum-free medium for 48 h and then protein levels of PI3K were determined by immunoblotting. B: Cells were pretreated with 100 or 500 nM wortmannin 1 h before being treated with 50 μg/ml rAgI/II-N and after 48 h of incubation, they were processed for the analysis of p-Akt levels. C: Osteoblasts exposed to 50 μg/ml rAgI/II-N and/or 100 nM wortmannin were processed for the MTT assay (C) or DNA synthesis determination (D) after 72-h incubation. $^{***}P < 0.001$ versus untreated cells. $^{\#}P < 0.05$ versus rAgI/II-N treatment alone.

and related mechanisms in the survival, proliferation, and differentiation of many types of cells [Cabodi et al., 2010; Heo and Lee, 2011; Pinon and Wehrle-Haller, 2011]. In particular, integrins are known as the predominant binding sites of AgI/II protein and the interaction between them mediates important signaling to regulate various cellular events [Nobbs et al., 2007; Engels-Deutsch et al., 2011]. Our findings indicate that integrins α5 and β1 are the initial transducers of rAgI/II-N-mediated signaling. This is consistent with a previous report that showed that adherence and internalization of *S. gordonii* to epithelial cells involved β1 integrin recognition by AgI/II protein polypeptides [Nobbs et al., 2007]. Although we did not provide direct evidence that rAgI/II-N bound to α5β1 integrins, we hypothesize that the recombinant protein interacted with β1 integrin expressed on osteoblasts, resulting in signal transduction.

This study shows that rAgI/II-N treatment increases the migration of cells into the G_2/M phase of cell cycle progression in serum-starved osteoblasts. This is considered to be closely related to the increased induction of cell cycle stimulating proteins. Cell cycle progression is achieved through a tightly controlled cascade of cellular events. The transition of cells from the G_0/G_1 phase to the S phase depends on CDKs, which form complexes with cyclin E and cyclin D_1 [Jirawatnotai et al., 2004; Martin et al., 2005]. These CDK/cyclin complexes phosphorylate key proteins and activate transcription factors that promote DNA synthesis [Sherr, 1996]. Our present data show that serum starvation resulted in growth inhibition with an attendant increase in expression of the cell cycle inhibitory proteins, p27 and p21. In contrast, rAgI/II-N treatment increased significantly TdR incorporation by osteoblasts in serum-starved cultures, where the induction of cyclin D1 and E was through to be involved.

The present study also demonstrates that rAgI/II-N-stimulated survival of osteoblasts is closely associated with the attenuation of mitochondrial stress. An important mitochondrial event during apoptosis is the reduction of MMP. MMP depletion is induced by the actions of Bcl-2 and Bax; depletion of MMP causes the cytoplasmic release of proapoptotic molecules from the mitochondria [Marfe et al., 2009; Cheng and Su, 2010]. In this study, serum-starved osteoblasts showed apoptosis-like cell death followed by mitochondrial alterations in Bcl-2 family proteins and the loss of MMP, which were significantly attenuated by rAgI/II-N treatment. In particular, treatment of cells with a PI3K inhibitor almost completely blocked the rAgI/II-N-mediated attenuation of mitochondrial stress. Akt inhibitor, GSK690693, also showed a significant inhibition on the protein-mediated increase of viability and DNA synthesis in primary osteoblasts (data not shown). We also found that rAgI/II-N stimulated Akt phosphorylation in osteoblasts incubated in medium containing 10% FBS (data not shown). These results lead us to postulate that PI3K/Akt phosphorylation is the pivotal event required for rAgI/II-N-mediated survival in serum-starved osteoblasts.

Akt is also believed to be involved in cell survival via inhibition of pro-apoptotic proteins and activation of anti-apoptotic proteins

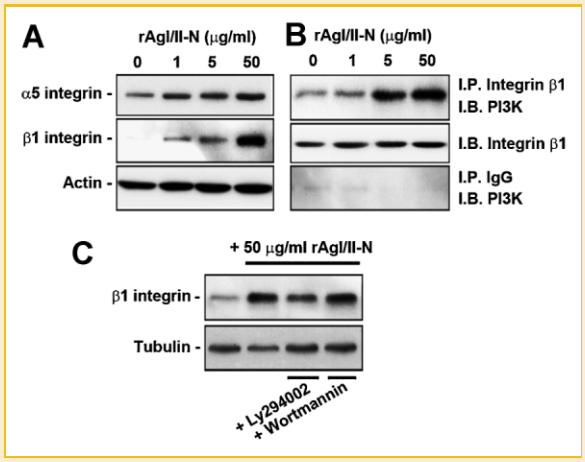

Fig. 8. Dose-dependent increase in expression of α5β1 integrin by rAgI/II-N in serum-starved osteoblasts. A: Cells were treated with increasing doses (0–50 μg/ml) of rAgI/II-N in serum-free medium and after 48 h of incubation, protein levels of integrin α5 and β1 were determined by immunoblotting. B: Immunoprecipitates from the same protein lysates using integrin β1 were also analyzed by Western blotting. C: In addition, cells were pretreated either with 10 μM Ly294002 or 100 nM wortmannin 1 h before the addition of 50 μg/ml rAgI/II-N. At 48 h after treatment, β1 integrin levels were measured by Western blotting.

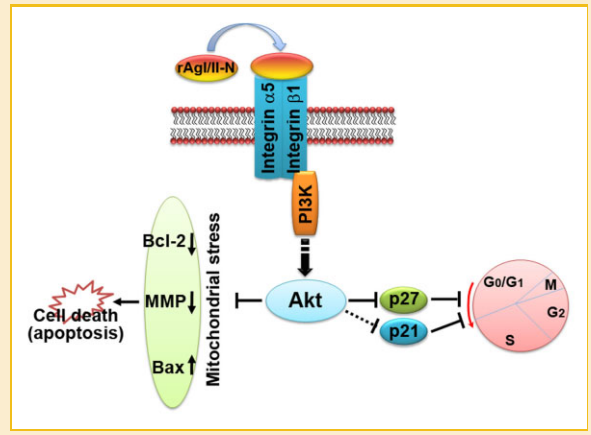

Fig. 9. Scheme of rAgI/II-N-mediated survival signaling in serum-starved osteoblasts. rAgI/II-N binds to the cell surface receptor, α5β1 integrin, which results in the phosphorylation of PI3K/Akt. PI3K/Akt in turn mediates survival in serum-starved osteoblasts by inhibiting mitochondrial stress and cell cycle disruption. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

[Hers et al., 2011]. Akt is a serine/threonine protein kinase that becomes fully active after dual phosphorylation of Thr^{308} and Ser^{473} [Pankov et al., 2003]. Integrins can act as positive modulators of Akt by stimulating its activation pathway involving the upstream kinase, PI3K [Watabe et al., 2011]. The PI3K/Akt pathway is also reported to play a role in inducing cell differentiation [Heo and Lee, 2011] and hypertrophy [Kornasio et al., 2009]. Further, Ras activation induces the phosphorylation of ERK and subsequently induces cell proliferation [Whelan et al., 2011]. In the present study, pharmacological inhibitors specific to PI3K, but not to ERK or Ras, had a negative effect on rAgI/II-N-mediated survival in serum-starved osteoblasts. We also found that Akt phosphorylation induced by rAgI/II-N in serum-starved osteoblasts was inhibited by addition of a PI3K inhibitor, whereas PI3K inhibitor did not affect the rAgI/II-N-mediated increase in levels of β1 integrin. Moreover, an immunoprecipitation assay showed a direct interaction between β1 integrin and PI3K after treatment of serum-starved osteoblasts with rAgI/II-N. These findings suggest that integrin-induced PI3K/Akt signaling pathways are closely related to rAgI/II-N-mediated survival signals in serum-starved osteoblasts (Fig. 9).

In conclusion, the current findings suggest that AgI/II protein modulates basic cellular events such as viability, proliferation, and survival in bone cells by activating integrin-linked kinases. In particular, phosphorylation of PI3K and Akt are important downstream events required for rAgI/II-N-induced integrin-mediated signaling in serum-starved osteoblasts. rAgI/II-N-mediated modulation on the cellular events appeared to be not limited only to the osteoblasts, because the proliferation of NIH3T3 fibroblasts was also stimulated by the protein. More detailed experiments in order to clarify the mechanisms by which AgI/II affects the cellular events and to explore a possibility of the protein in clinical use will be needed.

Additional experiments to clarify the roles of AgI/II on the inflammatory induction and the attendant bone resorption will be also needed.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0009123). Dr. J.-C. Lee was supported by Chonbuk National University for his sabbatical leave of absence at the University of Kentucky where he performed some of the experiments reported in this article. The authors sincerely thank Drs. Kyung-Yeol Lee and Sae-Hae Kim for their technical assistance and preparation of rAgI/II-N.

REFERENCES

Ara T, Kurata K, Hirai K, Uchihashi T, Uematsu T, Imamura Y, Furusawa K, Kurihara S, Wang PL. 2009. Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease. J Periodontal Res 44:21–27.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brady LJ, van Tilburg ML, Alford CE, McArthur WP. 2000. Monoclonal antibody-mediated modulation of the humoral immune response against mucosally applied *Streptococcus mutans*. Infect Immun 68:1796–1805.

Cabodi S, Di Stefano P, Leal Mdel P, Tinnirello A, Bisaro B, Morello V, Damiano L, Aramu S, Repetto D, Tornillo G, Defilippi P. 2010. Integrins and signal transduction. Adv Exp Med Biol 674:43–54.

Cheng CY, Su CC. 2010. Tanshinone IIA may inhibit the growth of small cell lung cancer H146 cells by up-regulating the Bax/Bcl-2 ratio and decreasing mitochondrial membrane potential. Mol Med Report 3:645–650.

Crowley PJ, Brady LJ, Michalek SM, Bleiweis AS. 1999. Virulence of a spaP mutant of *Streptococcus mutans* in a gnotobiotic rat model. Infect Immun 67:1201–1206.

Engels-Deutsch M, Pini A, Yamashita Y, Shibata Y, Haikel Y, Schöller-Guinard M, Klein JP. 2003. Insertional inactivation of pac and rmlB genes reduces the release of tumor necrosis factor alpha, interleukin-6, and interleukin-8 induced by *Streptococcus mutans* in monocytic, dental pulp, and periodontal ligament cells. Infect Immun 71:5169–5177.

Engels-Deutsch M, Rizk S, Haikel Y. 2011. *Streptococcus mutans* antigen I/II binds to α5β1 integrins via its A-domain and increases β1 integrins expression on periodontal ligament fibroblast cells. Arch Oral Biol 56:22–28.

Hajishengallis G, Sharma A, Russell MW, Genco RJ. 2002. Interactions of oral pathogens with toll-like receptors: Possible role in atherosclerosis. Ann Periodontol 7:72–78.

Han TK, Zhang C, Dao ML. 2006. Identification and characterization of collagen-binding activity in *Streptococcus mutans* wall-associated protein: A possible implication in dental root caries and endocarditis. Biochem Biophys Res Commun 343:787–792.

Heddle C, Nobbs AH, Jakubovics NS, Gal M, Mansell JP, Dymock D, Jenkinson HF. 2003. Host collagen signal induces antigen I/II adhesin and invasin gene expression in oral *Streptococcus gordonii*. Mol Microbiol 50:597–607.

Heo JS, Lee JC. 2011. β-Catenin mediates cyclic strain-stimulated cardiomyogenesis in mouse embryonic stem cells through ROS-dependent and integrin-mediated PI3K/Akt pathways. J Cell Biochem 112:1880–1889.

Hers I, Vincent EE, Tavaré JM. 2011. Akt signalling in health and disease. Cell Signal 23:1515–1527.

Jeon CW. Generation of a monoclonal antibody against AgI/II, a cellular surface protein of *Streptococcus mutans* GS5. Chonbuk National University. Ph.D thesis 2006. p 5–10.

Jeong JW, Kim JG, Baik BJ, Yang YM, Seo JA. 2005. Identification of the *agI/II* and *gtfD* genes from *Streptococcus mutans* GS-5. J Kor Acd Ped Dent 32:357–369.

Jirawatnotai S, Aziyu A, Osmundson EC, Moons DS, Zou X, Kineman RD, Kiyokawa H. 2004. Cdk4 is indispensable for postnatal proliferation of the anterior pituitary. J Biol Chem 279:51100–51106.

Keely PJ. 2011. Mechanisms by which the extracellular matrix and integrin signaling act to regulate the switch between tumor suppression and tumor promotion. J Mammary Gland Biol Neoplasia 16:205–219.

Kitten T, Munro CL, Wang A, Macrina FL. 2002. Vaccination with FimA from *Streptococcus parasanguis* protects rats from endocarditis caused by other *viridans streptococci*. Infect Immun 70:422–425.

Kornasio R, Riederer I, Butler-Browne G, Mouly V, Uni Z, Halevy O. 2009. Beta-hydroxy-beta-methylbutyrate (HMB) stimulates myogenic cell proliferation, differentiation and survival via the MAPK/ERK and PI3K/Akt pathways. Biochim Biophys Acta 1793:755–763.

Li JX, Fu LJ, Yang XG, Wang K. 2011. Integrin-mediated signaling contributes to gadolinium-containing-particle-promoted cell survival and G(1) to S phase cell cycle transition by enhancing focal adhesion formation. J Biol Inorg Chem In press.

Liang S, Hosur KB, Domon H, Hajishengallis G. 2010. Periodontal inflammation and bone loss in aged mice. J Periodontal Res 45:574–578.

Loesche WJ. 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 50:353–380.

Marfe G, Tafani M, Indelicato M, Sinibaldi-Salimei P, Reali V, Pucci B, Fini M, Russo MA. 2009. Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction. J Cell Biochem 106:643–650.

Martin A, Odajima J, Hunt SL, Dubus P, Ortega S, Malumbres M, Barbacid M. 2005. Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27(Kip1) and p21(Cip1). Cancer Cell 7:591–598.

Murakami Y, Nakano Y, Yamashita Y, Koga T. 1997. Identification of a frameshift mutation resulting in premature termination and loss of cell wall anchoring of the PAc antigen of *Streptococcus mutans* GS-5. Infect Immun 65:794–797.

Nakano K, Tsuji M, Nishimura K, Nomura R, Ooshima T. 2006. Contribution of cell surface protein antigen PAc of *Streptococcus mutans* to bacteremia. Microbes Infect 8:114–121.

Nobbs AH, Shearer BH, Drobni M, Jepson MA, Jenkinson HF. 2007. Adherence and internalization of *Streptococcus gordonii* by epithelial cells involves beta1 integrin recognition by SspA and SspB (antigen I/II family) polypeptides. Cell Microbiol 9:65–83.

Pankov R, Cukierman E, Clark K, Matsumoto K, Hahn C, Poulin B, Yamada KM. 2003. Specific beta1 integrin site selectively regulates Akt/protein kinase B signaling via local activation of protein phosphatase 2A. J Biol Chem 278:18671–18681.

Pinon P, Wehrle-Haller B. 2011. Integrins: Versatile receptors controlling melanocyte adhesion, migration and proliferation. Pigment Cell Melanoma Res 24:282–294.

Robinette RA, Oli MW, McArthur WP, Brady LJ. 2011. A therapeutic anti-*Streptococcus mutans* monoclonal antibody used in human passive protection trials influences the adaptive immune response. Vaccine 29:6292–6300.

Sherr CJ. 1996. Cancer cell cycles. Science 274:1672–1677.

Watabe H, Furuhama T, Tani-Ishii N, Mikuni-Takagaki Y. 2011. Mechanotransduction activates α(5)β(1) integrin and PI3K/Akt signaling pathways in mandibular osteoblasts. Exp Cell Res 317:2642–2649.

Whelan JT, Hollis SE, Cha DS, Asch AS, Lee MH. 2011. Post-transcriptional regulation of the Ras-ERK/MAPK signaling pathway. J Cell Physiol In press.

Zhao Y, Qiu F, Xu S, Yu L, Fu G. 2011. Thymosin β4 activates integrin-linked kinase and decreases endothelial progenitor cells apoptosis under serum deprivation. J Cell Physiol 226:2798–2806.