The effect of different surface roughnesses on the differentiation of MC3T3-E1 mouse osteoblasts *in vitro*

Kiyoto Takemoto¹, Kenichi Matsuzaka^{2,3}, Masaki Shimono¹, Masao Yoshinari³, Takashi Inoue^{2,3}

¹Department of Pathology, Tokyo Dental College, Chiba, Japan

²Oral Health Science Center hrc7, Tokyo Dental College, Chiba, Japan

³Department of Clinical Pathophysiology, Tokyo Dental College, Chiba, Japan

Abstract:

Background: The purpose of this study was to investigate the differentiation and maturation of osteoblasts on surfaces with different roughnesses.

Methods: Blasting a culture dish surface with Al₂O₃ particles of 25 μm, 50 μm or 180 μm diameter produces mean surface a roughness (Ra) of 0.48 ± 0.07, 1.02 ± 0.14 or 3.10 ± 0.36, respectively. Morphological changes of cells were compared, and the expression of mRNAs of osteopontin and osteocalcin were evaluated. Further, the ratio of alizarin red positive area was calculated.

Results: Cells cultured on the 180 μm blasted surface had almost the same morphology as those cultured on the 50 μm blasted surface; however, cellular bridges tended to be much wider and more spread out. The expressions of osteopontin and osteocalcin mRNAs in cells cultured on the 180 μm blasted surface have greater up-regulation than the other cells. Further, the ratio of alizarin red positive area in 180 μm blasted surface was significantly higher than those of others.

Conclusions: These results suggest that the 180 μm blasted surface, with a surface roughness of approximately 3 μm in width, is the best surface for MC3T3-E1 cells to differentiate and mature *in vitro*.

[Oral Med Pathol 2010; 15: 45-51 doi: 10.3353/omp.15.45]

Key words: surface geometry, osteoblast, SEM, mRNA expression

Correspondence: Kenichi Matsuzaka, Oral Health Science Center hrc7 Department of Clinical Pathophysiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502 Japan
Phone: 81-43-270-3581, Fax: 81-43-270-3583, E-mail: matsuzak@tdc.ac.jp

Introduction

Many studies have compared the influence of surface geometry and the characteristics of dental implants on cell behavior using *in vivo* and *in vitro* model systems (1-18). Dental implant surface characteristics can markedly influence cell attachment, orientation and subsequent osseointegration *in situ*. In particular, osteoblastic cell differentiation and maturation around titanium dental implants have been widely studied (4, 13, 15, 19). Those studies show that titanium implant surfaces may modulate the phenotypic expression and metabolism of osteoblastic cells (2, 20). However, published studies on the biological effects of surface properties on osteoblastic cell differentiation and maturation do not reveal whether different surfaces are more suitable than others.

Differentiation toward an osteoblastic phenotype is a multi-step process which is regulated by several factors, such as hormones and growth factors (21) and also by surface geometry (14). The morphological changes

undergone by cells during attachment and flattening onto a matrix surface have been described by Taylor (17). In Taylor's model, the cells adhere to the substratum and progressively flatten on the substrate. These changes in cell morphology are also related to cell differentiation in terms of their function as mechanoreceptors, and in terms of the pathway which might link the nucleus to activate the proper osteoblastic gene, such as osteopontin (OPN) or osteocalcin (OCN).

In cell cultures, it is difficult to recognize the differentiation and maturation of cells using only morphological change as a criterion. The study of mRNA expression has recently developed as a useful tool to investigate weak cell signals. To detect the weak signal of OPN, which is known to be related to osteogenesis, and OCN, which is known to be related to calcification, the reverse transcription polymerase chain reaction (RT-PCR) method is quite useful.

In this study, the morphology of cells attached to different surfaces created by blasting with different sized

Al₂O₃ particles was investigated using scanning electron microscopy (SEM). Furthermore, the expression of mRNAs encoding 2 bone related proteins, OPN and OCN, was also evaluated using RT-PCR.

Materials and methods

Surface preparation by blasting

Cells surrounding dental implants include fibroblasts and osteoblasts, whose cell sizes are known to be 20 to 50 μ m. Furthermore, osteoclasts are multinucleated giant cells, with a diameter ranging anywhere from 100 to 200 μ m. Accordingly, the substrates used in this study had three different kinds of surface roughness. The internal surfaces of culture dishes were blasted with Al₂O₃ (aluminum oxide), with grain sizes of 25 μ m, 50 μ m, or 180 μ m diameter, at a pressure of 0.5 MPa. Each surface roughness was then characterized using a profilometer (Handysurf E-30A, Tokyo Seimitsu, Tokyo). Measurements were done on 3 randomly selected fields of each sample, and the mean surface roughness (Ra), the maximum surface roughness (Rmax) and the spacing between the peaks (Sm) were calculated. Untreated 35 mm culture dishes were used as controls.

Preparation of cell suspensions

MC3T3-E1 osteoblast-like cells derived from mice (Riken, Tokyo, Japan) were cultured with α -MEM (minimum essential medium, Gibco, Carlsbad, CA, USA) with 10% fetal calf serum supplemented 10,000 M/ml penicillin G and 10,000 μ g/ml streptomycin sulfate. Furthermore, 10⁻⁸ M dexamethasone and 10 mM β -glycerophosphate were added to the medium. Cells were cultured in 5% (v/v) carbon dioxide (CO₂) at 37°C for 5 days until they were confluent, with changes of medium every 48 hrs. Cells were observed every day by phase-contrast microscopy. When the cells became confluent, they were detached from the flasks using 0.1% trypsin. The cell number in each suspension was determined and adjusted to 2 $\times$ 10⁴ cells/ml in the same culture medium. Approximately 4 $\times$ 10⁵ cells in 5 ml media were seeded on each different surface of culture dish described above. After 12 hrs incubation at 37°C, the cells were washed with phosphate-buffered saline (PBS) to remove any unattached cells.

Fig. 1. Scanning electron microscopy (SEM) image about the proportion of area of cellular bridge (PACB). The PACB (arrow) in the whole cell area was calculated on the SEM image.

Scanning electron microscopic evaluation of cells

Cells cultured on each surface were fixed with 2.5% glutaraldehyde in 0.1 mM sodium cacodylate buffer (pH 7.2), at 3, 7 and 10 days of culture. Cells were washed with PBS, and then dehydrated in graded ethanols and critical point-dried from liquid CO₂ using a critical point-drier. Samples were sputter coated with gold for 2 min and were viewed using a HITACHI 8000 SEM.

The proportion of area of cellular bridge (PACB) in the whole cell area was calculated on the SEM image (Fig.1),

$$\text{PACB (\%)} = \frac{\text{area of cellular bridge}}{\text{all area of cells}} \times 100$$

RT-PCR

Total RNAs were extracted using the Isogen reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Briefly, cultured osteoblast-like cells were homogenized and solubilized in an Isogen/chloroform solution at 4°C. Supernatants were obtained by centrifugation at 12,000 $\times$ g for 20 min at 4°C. The precipitates were obtained by decantation and were washed with 75% ethanol. Finally, the RNA pellets were dissolved in RNAase-free water and stored at -20°C until used. Using the extracted RNA as a template, reverse transcription reactions were carried out with an RT-PCR kit (RNA PCR

Table 1. PCR primers used for PCR analysis

Target cDNA	Primer sequence	PCR condition (cycle anealing)	Product size
osteopontin	Forward (5'-CTC GGA GGA GAA GGC GCA TTA -3')	45 cycles, 95°C (10s), 66°C (10s), 72°C (10s)	207 bp
	Reverse (5'-CCA TCG TCA TCG TCG TCG TCA -3')		
osteocalcin	Forward (5'-GGT GCA AAG CCC AGC GAC TCT -3')	45 cycles, 95°C (10s), 56°C (5s), 72°C (9s)	199 bp
	Reverse (5'-GGA AGC CAA TGT GGT CCG CTA -3')		
GAPDH	Forward (5'-TGAACGGGAAGCTCACTGG-3')	40 cycles, 95°C (10s), 60°C (10s), 72°C (12s)	307 bp
	Reverse (5'-TCCACCACCTGTGTGCTGTA-3')		

Table 2. Surface roughness produced by blasting a smooth culture dish surface with Al_2O_3 particles

particle size		mean surface roughness (μm)	maxim surface roughness (μm)	spacing between the peaks (μm)
25 μm	AV	0.48	5.82	23.4
	SD	0.07	1.3	2.62
50 μm	AV	1.02	9.58	37.9
	SD	0.14	0.9	6.3
180 μm	AV	3.1	28.9	57.1
	SD	0.36	5.41	6.42

kit ver 2.1, Takara Bio Inc., Kyoto, Japan) to synthesize cDNA. PCR was then carried out for 35 cycles using OPN and OCN primers (Table 1). PCR products were separated on 2% agarose ethidium bromide gels. Data were normalized using the ratio of sample data to that of the housekeeping gene glucose-6-phosphate dehydrogenase (GAPDH). After normalization, mRNA levels were expressed relative to GAPDH.

To determine mRNA levels, a quantitative RT-PCR assay was conducted using a LightCycler with the double-stranded DNA dye SYBR Green I (F. Hoffmann-La Roche Ltd, Basel, Switzerland). The primers used for the LightCycler assays were the same as those used for ordinary RT-PCR. Quantification was performed by comparing the levels obtained to standardized samples. In the present study, the concentrations of cDNA in unstimulated samples were 0.2, 0.5, 1.0 and 2.0 μl . The PCR conditions used in the LightCycler are 40 cycles (95°C 10 sec, 60°C 5 sec, 72°C 12 sec). Melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products.

Alizarin red staining and ratio of positive area

Cells cultured on each surface were fixed with 10% formalin after 2 weeks of culture. Cells were washed with PBS and then immersed in 1% alizarin red solution (pH 7.0, sodium alizarin sulfonate, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then the ratios of alizarin red positive areas were calculated.

Statistical analysis

The results were analyzed via one-way ANOVA and were then compared by the Scheffe's test. The level of significance was 0.05 and 0.01 in these tests.

Results

Surface roughness

Blasting a smooth culture dish surface with Al_2O_3 particles of 25 μm , 50 μm or 180 μm diameter produced a mean surface roughness (R_a) of $0.48 \pm 0.07 \mu\text{m}$, $1.02 \pm 0.14 \mu\text{m}$ or $3.10 \pm 0.36 \mu\text{m}$, respectively; a maximum surface roughness (R_t) of $5.82 \pm 1.30 \mu\text{m}$, $9.58 \pm 0.90 \mu\text{m}$ or $28.90 \pm 5.41 \mu\text{m}$, respectively; and a spacing between peaks (S_m) of $23.40 \pm 2.62 \mu\text{m}$, $37.90 \pm 6.30 \mu\text{m}$ or $57.10 \pm 6.42 \mu\text{m}$, respectively (Table 2). These surfaces were observed to be hollow with specific dimensions and shapes. Fig. 1 shows

the SEM pictures of blasted culture dishes.

Cell morphology on different surface roughnesses

Cells cultured on untreated 35 mm culture dishes were characterized as spindle-shaped with many microspikes at all time periods (Fig. 2). Cells cultured on the 25 μm blasted surface were spindle-shaped at day 3, and were spread out with processes at days 7 and 10. The cells had adapted to the underlying topography at all time periods (Figs. 3 a-c). Cells cultured on the 50 μm blasted surface grew deep into the hollows and had attached with spikes to the substrate. Slender cell bridges were frequently observed at day 3.

The cells were scattered widely and had attached to the substrate with processes at days 7 and 10 (Figs. 3 d-f).

At day 10, the cells had adapted to the underlying topography. Cells cultured on the 180 μm blasted surface made much wider cellular bridges than did cells cultured on the 50 μm blasted surface, and had multiple points of attachment to the substrate at all time periods (Figs. 3 g-i). At day 10, the cells had adapted to the underlying topography.

Proportion of area of cellular bridge (PACB)

PACB on the 25 μm surface was the lowest and on the 180 μm surface was the highest at any of the time periods with a significant difference ($P < 0.01$). PACB on the 50 μm surface was just between 25 μm and 180 μm without significant difference (Fig. 4).

Fig. 2. SEM image of MC3T3-E1 cells on smooth surface. Arrowheads show cells attaching to the surface.

Fig. 3. SEM images of MC3T3-E1 cells. (a) 3 days on 25 μm blasted surface; (b) 7 days on 25 μm blasted surface; (c) 10 days on 25 μm blasted surface; (d) 3 days on 50 μm blasted surface; (e) 7 days on 50 μm blasted surface; (f) 10 days on 50 μm blasted surface; (g) 3 days on 180 μm blasted surface; (h) 7 days on 180 μm blasted surface; (i) 10 days on 180 μm blasted surface. On the 25 μm blasted surface, cells had adapted to the underlying topography during the experimental period (a-c). On the 50 μm blasted surface, cells grew deep into the hollows, and were widely spread and had attached to the substrate with processes at days 7 and 10 (d-f). On the 180 μm blasted surface, cells make a much wider cellular bridges (g-i).

Fig. 4. The proportion of PACB areas in MC3T3-E1 cells cultured on different surface conditions. □, 25 μm surface; ▒, 50 μm surface; ■, 180 μm surface. PACB on the 25 μm surface was the lowest and on 180 μm surface was the highest at any of the time periods with significant difference ($P < 0.01$). PACB on the 50 μm surface was just between 25 μm and 180 μm without significant difference.

Fig. 5. Expression of osteopontin mRNA in MC3T3-E1 cells cultured on different surface conditions. □, control; ▒, 25 μm surface; ▒, 50 μm surface; ■, 180 μm surface. Expression of OPN/GAPDH mRNA ratio in cells cultured on the 180 μm blasted surface show higher up-regulation compared to those of control and 25 μm blasted surfaces at day 3 ($P < 0.01$) and all others at day 7 ($P < 0.01$). However, there was no significant difference among any of the groups at day 10. (** $P < 0.01$)

Expression of mRNAs

Expression of OPN mRNA in cells cultured on the 180 μ m blasted surface had higher up-regulation compared to that in cells cultured on the control and the 25 μ m blasted surfaces at day 3 ($P<0.01$) and all others at day 7 ($P<0.01$). However, there was no significant difference among any of the groups at day 10 (Fig. 5).

Expression levels of OCN mRNA in cells cultured on the 180 μ m blasted surface had higher up-regulation compared to those in control cells at day 3 ($P<0.05$). However, there was no significant difference among any of the groups at days 7 or 10 (Fig. 6).

The ratio of alizarin red staining positive area

The ratio of alizarin red staining positive area was significantly higher for the 180 μ m blasted surfaces than for those of the control, 25 μ m and 50 μ m blasted surfaces (Fig. 7).

Discussion

The method used in this study was similar to a technique previously described, which compared initial cellular responses to implants of different surface characteristics (5–6). Several studies (12, 14) have described sequential morphologic changes of osteoblast-like cells as a function of time and have provided detailed descriptions of cellular morphology. However, few studies have investigated osteogenic cellular differentiation and maturation in terms of morphology and expression of mRNAs encoding bone related proteins.

Matsuzaka *et al.* (11) reported that microgrooves could control osteogenic cell orientation and differentiation and could enhance the production of mineralized matrix *in vitro*. They examined different sizes of microgrooves and found better cell orientation on microgrooves 1 μ m to 2 μ m in

width and detected accelerated calcification. However, cells cultured on wider microgrooves of either 5 μ m or 10 μ m in width attached to the substrate just the same as on smooth surfaces, and there was no acceleration of calcification. Loesberg *et al.* (9,10) used the same size microgrooves as Matsuzaka *et al.* (11) and suggested that cell differentiation was accelerated on microgrooves of 1 μ m to 2 μ m in width, because cells were suspended in the groove as a cellular bridge and exposed receptors existing on the cell membrane, making it easier to get molecular signals such as dexamethasone into the culture medium and cause (or induce) the cells to differentiate into mature osteoblasts.

In this study, we blasted culture dish surfaces with Al_2O_3 particles of 25 μ m or 50 μ m diameter to produce mean surface roughnesses (Ra) of 0.48 ± 0.07 or 1.02 ± 0.14 , respectively. The expressions of mRNAs encoding 2 bone related proteins (OCN and OPN) had not up-regulated compared to those of the control group in this study.

Those surface roughnesses were not significantly different from the untreated surface and might not be effective to induce differentiation. However, the 180 μ m blasted surface produced a mean surface roughness (Ra) of 3.10 ± 0.36 which was wider than the 1 to 2 μ m microgrooves described above, but the effect was just the same as those microgrooves. However, the cells adapted to the underlying topography at later time periods even on the 180 μ m blasted surface without making cellular bridges. This might be the reason why the expression of bone-related protein mRNAs had up-regulated at early time periods, but not at late time periods.

OPN is an Arg-Gly-Asp-containing noncollagenous bone protein whose mRNA is found during the stages of bone formation and bone remodeling (22). OPN has been hypothesized to be not only an osteoblast-associated gene, but also a multifunctional cytokine that generates intracellular signals produced by various bone-related cells and other

Fig. 6. Expression of osteocalcin mRNA in MC3T3-E1 cells cultured on different surface conditions. □, control; ▤, 25 μ m surface; ▥, 50 μ m surface; ■, 180 μ m surface. Expression levels of OCN/GAPDH mRNA ratio in cells cultured on the 180 μ m blasted surface show higher up-regulation compared to those of controls at day 3 ($P<0.05$). However, there was no significant difference among any of the groups at days 7 or 10 (* $P<0.05$).

Fig. 7. The ratio of alizarin red staining positive area in MC3T3-E1 cells cultured on different surface conditions. □, control; ▤, 25 μ m surface; ▥, 50 μ m surface; ■, 180 μ m surface. The ratio of positive area in 180 μ m blasted surface was significantly higher than those on the control, 25 μ m, and 50 μ m blasted surfaces (** $P<0.01$).

sorts of cells (11). Toma *et al.* (23) demonstrated that OPN produced by osteoblasts plays a role in the mechanosignal transduction that requires microfilament structures for signal transduction, and this possibly depends on a protein kinase A-like kinase. OCN is produced only by osteoblasts and has an affinity for bone mineral constituents (24) which implies a role in mineralization of the bone matrix during final osteoblast differentiation (11). Furthermore, the OCN gene has an activator protein-1 (AP-1) binding site (25), and AP-1 (including the *c-fos* gene transcription complex) is known to be a strong regulator of osteoblastic proliferation and differentiation. Down-regulation of OCN probably reduces *c-fos* levels, which would also suppress bone formation. Thus, the higher expression of both OPN and OCN at early time periods in cells cultured on the 180 μm blasted surface suggests that the 3 μm Sa is a critical size for MC3T3-E1 cells in terms of differentiation and maturation for bone producing cells. On the other hand, it is well known that alizarin red staining can be detected mineralized tissue. In our study, the ratio of alizarin red staining positive area for the 180 μm blasted surface was higher than for the others. This also indicated that the 180 μm blasted surface causes MC3T3-E1 cells to be differentiated and to mature.

Salter *et al.* demonstrated that integrins, which link components of the extracellular matrix with actin in the cytoplasm, function as mechanoreceptors (26), and Tanaka suggested that this pathway might link the nucleus to mechano-transduction (27). It is known that mechanical force, including microgravity at areas of cellular bridges because of surface geometry, affects cellular morphology as a result of changes in actin filament arrangement and promotes cellular differentiation due to the mechano-transduction, as described above. In this study, the surface roughness created by larger particles, such as ones that are 180 μm in diameter, possibly affects the structural pathway. However, the maximum surface roughnesses (R_t) were 5.82 ± 1.30 , 9.58 ± 0.90 , and 28.90 ± 5.41 , and the spacings between the peaks (S_m) were 23.40 ± 2.62 , 37.90 ± 6.30 and 57.10 ± 6.42 .

These huge sizes of R_t and S_m are too large for the cells and probably are not effective to induce cell differentiation.

Taken together, cells cultured on the 180 μm blasted surface demonstrate that a 3 μm Sa in width is a critical size to induce MC3T3-E1 cells to differentiate and mature into osteoblasts. This probably results from both mechano-transduction of intranuclear genes and exposure of surface receptors for hormones, such as dexamethasone.

In conclusion, the surface roughness created by blasting using 180 μm Al_2O_3 seems an appropriate surface for MC3T3-E1 cells to differentiate in early stage *in vitro*.

Acknowledgments

The authors would like to thank Ms. Saori Takano for her technical assistance. This research was supported by an Oral Health Science Center Grant HRC7 from the Tokyo Dental College, and by a "High-Tech Research Center" Project for Private Universities: matching fund subsidy from

MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2006-2010.

References

1. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast responses in vitro. *Int J Oral Maxillofac Implants* 1992; **7**: 302-10.
2. Brunette DM. The effect of implant surface topography on the behavior of cells. *Int J Oral Maxillofac Implants* 1988; **3**: 231-46.
3. Chang Y-L, Stanford CM, Wefel JS, Keller JC. Osteoblastic cell attachment to hydroxyapatite-coated implant surfaces in vitro. *Int J Oral Maxillofac Implants* 1999; **14**: 239-47.
4. Davies JE. Mechanism of endosseous integration. *Int J Prosthodont* 1998; **11**: 391-401.
5. Inoue T, Matsuzaka K, Yoshinari M, Tanaka T, Abiko Y, Shimono M. Current Dental Implant Research. *Dentistry in Japan* 2005; **41**: 196-213.
6. Inoue T, Matsuzaka K, Yoshinari M, Abiko Y, Shimono M. Implant-bone Tissue Interface. *Bull Kanagawa Dent Coll* 1999; **27**: 132-41.
7. Keller JC, Stanford CM, Wightman, JP, Draughn, RA, Zaharias R Characterizations of titanium implant surfaces. *J Biomed Mater Res* 1994; **28**: 939-46.
8. Kieswetter K, Schwartz Z, Dean DD, Boyan BD. The role of implant surface characteristics in the healing of bone. *CRC Crit Rev Oral Biol Med* 2000; **7**: 329-45.
9. Loesberg WA, Walboomers XF, Bronkhorst EM, van Loon JJ, Jansen JA. The effect of combined simulated microgravity and microgrooved surface topography on fibroblasts. *Cell Motil Cytoskeleton* 2007; **64**: 174-85.
10. Loesberg WA, Walboomers XF, van Loon JJ, Jansen JA. The effect of combined hypergravity and micro-grooved surface topography on the behavior of fibroblasts. *Cell Motil Cytoskeleton* 2006; **63**: 384-94.
11. Matsuzaka K, Yoshinari M, Shimono M, Inoue T. Effects of multi-grooved surfaces on osteoblast-like cells in vitro: Scanning electron microscopic observation and mRNA expression of osteopontin and osteocalcin. *J Biomed Mater Res* 2004; **68A**: 227-34.
12. Nashimoto M, Matsuzaka K, Yoshinari M, Shimono M, Inoue T. The effect of surface pore size on the differentiation of rat bone marrow cells: Morphological observations and expression of bone related protein mRNA. *Bull Tokyo Dental Coll* 2005; **45**: 201-11.
13. Schwartz Z, Boyan BD. Underlying mechanisms at the bone-biomaterial interface. *J Cell Biochem* 1994; **56**: 340-7.
14. Shiigai T, Shimono M, Yoshinari M, Matsuzaka K, Inoue T. The effect of substrate pore size on the differentiation of L929 fibroblast cells: Morphological observations and expression of type 1 collagen mRNA. *Biomedical Res* 2004; **25**: 61-8.
15. Swart KM, Keller KC, Wightma JP, Draughn RA, Stanford CM, Michaels CM. Short-term plasma-cleaning treatments enhance in vitro osteoblast attachment to titanium. *J Oral Implantol* 1992; **18**: 130-7.
16. Sykaras N, Iacopino AM, Marker VA, Triplett RG, Woody

- RD. Implant materials, design, and surface topographies: Their effect on osseointegration. A literature review. *Int. J Oral Maxillofac Implants* 2000; **15**: 625-90.
17. Taylor AC. Adhesion of cells to surface. In: Manly RS ed. *Adhesion in Biological systems*. Academic Press, New York, 1970; 51-71.
18. Vezeau PJ, Koorbusch GF, Draughn RA, Keller JC Effects of multiple sterilization on surface characteristics and in vitro biologic responses to titanium. *J Oral Maxillofac Surg* 1996; **54**: 738-46.
19. Stanford CM, Keller JC, Solursh M. Bone cell expression on titanium surfaces is altered by sterilization treatment. *J Dent Res* 1994; **73**: 1061-71.
20. Cooper LF, Tiffie JC, Griffin J P, Hamano H, Guo Z. Estrogen-induced resistance to osteoblast apoptosis is associated with increased hsp27 expression. *J Cell Physiol* 2000; **185**: 401-7.
21. Lian JB, Stein GS. The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF β 1) and hormones (Vitamin D and glucocorticoids). *J Oral Implantol* 1993; **19**: 95-105.
22. Denhardt DT, and Guo X. Osteopontin: a protein with diverse functions. *Gaseb* 1993; **J7**: 1475-82.
23. Toma CD, Ashr S, Gray ML, Schaffer JL Gerstenfeld LC. Signal transduction of mechanical stimuli is dependent on microfilament integrity; identification of osteopontin as a mechanically induced gene in osteoblasts. *J Bone Miner Res* 1997; **12**: 1626-36.
24. Fujii K, Inoue H. *Biology of Bone and Cartilage Application of Basic Science for Practice Medicine*. Kanehara, Tokyo, 2002; 25-7.
25. Bilezikian JP, Raisz LG, Rodan GA. *Principles of Bone Biology*. Academic Press, San Diego, 1996; 77-9.
26. Salter DM, Robb J, Wright MO. Electrophysiological responses of human bone cells to mechanical stimulation; evidence for specific integrin function in mechanotransduction. *J Bone Miner Res* 1997; **12**: 1134-41.
27. Tanaka H. Mechanical stress and bone resorption. *Mol Med* 2001; **38**: 656-60.