Factors Influencing Effects of Specific COX-2 Inhibitor NSAIDs on Growth and Differentiation of Mouse Osteoblasts on Titanium Surfaces

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***Purpose**: To investigate the influence of exposure time and stages of cell growth on the effects of specific COX-2 inhibitor NSAIDs on growth and differentiation of osteoblasts on smooth titanium surfaces.* ***Materials and Methods:** The study was categorized into 5 groups: group A, 0.1 μM indomethacin; group B, 1.5 μM celecoxib; group C, 3.0 μM celecoxib; group D, 9.0 μM celecoxib; and group E, serum-free culture medium without drug treatment. A mouse calvarial cell line, MC3T3-E1, was seeded on acid-prickled surface titanium disks. The investigations were performed in 3 experimental phases based on stages of cell growth: static (24 hours after seeding), log (culture day 5), and plateau (culture day 12). In each experimental phase, cells on titanium disks were incubated in a medium treated with drugs according to the groups of study for 1, 3, and 5 days.* ***Results:** Indomethacin and celecoxib in groups A to D inhibited growth of cells on treatment days 3 and 5 in static phase and on treatment day 3 in log phase. Additionally, an inhibitory effect of indomethacin was greater than celecoxib. Effects on alkaline phosphatase (ALP) activity and osteocalcin were not clearly demonstrated. A significant decrease of PGE_2 production was found in groups A to D in static and plateau but not log phases.* ***Conclusion:** A specific COX-2 inhibitor NSAID, celecoxib, inhibited growth of osteoblasts on titanium surfaces and the effects were influenced by exposure time and stages of cell growth. Using a specific COX-2 inhibitor might cause deterioration of osteointegration of dental implants by interfering with osteoblastic cell growth in the proliferative stage.* Int J Oral Maxillofac Implants 2008;23:1071–1081

Key words: *growth and differentiation, osteoblasts, specific COX-2 inhibitor NSAIDs, titanium surface*

The specific COX-2 inhibitor NSAID, celecoxib, is an analgesic drug for the management of chronic and acute pain. It was developed to avoid the adverse effects of nonspecific cyclooxygenase 1 and 2 (COX-1 and COX-2) inhibitor NSAIDs caused by inhibition of the constitutive function of COX-1.[1,2] Because COX-2 is an enzyme produced in response to stress and trauma,[1] specific COX-2 inhibitor NSAIDs may inhibit the inflammatory process or production of prostaglandins necessary for bone healing and metabolism[3,4] in the early stage of wound healing and repair.

Prostaglandin E2 (PGE_2) stimulates bone remodeling by promoting both anabolic and catabolic responses depending on the target cell population and the concentration of PGE_2.[5,6] PGE_2 acts through G-protein-coupled cell-surface receptors, EP1-EP4, which are expressed by osteoblasts and pre-osteoblasts.[7] When PGE_2 is administrated, it promotes recruitment and osteoblastic differentiation of bone marrow osteogenic precursor cells resulting in significant increases in bone mass, strength and density, and rate of new bone formation.[5,8,9] Prostaglandin EP4 agonist augmented the osteoconductivity and improved the stability of the implant-bone attachment of hydroxyapatite-coated implants in the osteoporotic rat model.[10]

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Evidence from animal studies demonstrates that COX-2 inhibitors inhibit bone cell proliferation and delay fracture healing in dose- and time-dependent manners.[11,12] The roles of COX-2 in osteogenesis and bone metabolism are emphasized by Zhang et al[13]: COX-2 plays an essential role in endochondral and intramembranous bone formation during skeletal repair and osteoblastogenesis of mesenchymal stem cells in bone marrow. Inhibitory effects of specific COX-2 inhibitors in vivo are supported by in vitro studies showing that specific COX-2 inhibitors and conventional NSAIDs inhibit proliferation of human osteoblasts on the plastic surface of cell culture plates[14] and decrease differentiation of osteoblasts on rough titanium surfaces.[15] Further studies are required to demonstrate the effects of specific COX-2 inhibitor NSAIDs on osteointegration of implants, particularly in the use of high doses and/or extended periods of time, such as in osteoarthritis and rheumatoid arthritis cases, in which celecoxib must be taken for at least 2 weeks.[16]

For the current study, the authors intended to examine factors influencing adverse effects of specific COX-2 inhibitor NSAIDs on osteointegration of dental implants by investigating dose- and exposure time–dependent effects of a specific COX-2 inhibitor on growth and differentiation of osteoblasts on acid prickled titanium surfaces in different stages of cell growth in vitro. It was hypothesized that inhibitory effects of specific COX-2 inhibitor NSAIDs on growth and differentiation of osteoblasts were influenced by doses, exposure time, and stages of cell growth, and the effects might be subsequently an inhibitory effect of cyclooxygenase enzymes on the production of PGE_2.

MATERIALS AND METHODS

Study Groups

The study was categorized into 5 groups based on drugs in the culture medium: group A, 0.1 μM indomethacine; group B, 1.5 μM celecoxib; group C, 3.0 μM celecoxib; group D, 9.0 μM celecoxib; and group E, culture medium without drug treatment.

Preparation of Titanium Disk for Cell Seeding

Commercially pure titanium disks with an acid prickled surface, 15 mm in diameter and 1 mm thick, were provided by Institut Straumann (Straumann, Basel, Switzerland). Disks were prepared for cell seeding according to Lohmann et al.[17] Prior to cell seeding, disks were sonicated (Cavitator ME11, Mettler Electronics, Anaheim, CA, USA), rinsed in double distilled water, neutralized in 5% sodium bicarbonate, and rinsed in deionized water. The disks were then air dried and sterilized by autoclaving. Disks were placed in a 24-well culture plate, 1 disk in 1 well for cell culture on a titanium surface.

MC3T3-E1 Cell Culture

The MC3T3-E1 mouse cell line was obtained from ATCC (USA). The cell line throughout the cell culture period was cultivated in complete culture medium composed of alpha-MEM medium, 10% FBS, 1% penicillin/streptomycin, 0.5% Fungizone and 50 μg/mL ascorbic acid. Culture medium was changed every 2 days.[18] Cells were cultivated into the fourth, fifth, and sixth passages. When the growth of cells reached 80% confluence, cells were trypsinized and seeded on titanium disks, 4×10^4 cells in 200 μL of culture medium per disk. The seeded cells were allowed to attach on the disks in a minimum culture medium for 3 hours in 5% CO_2 at 37°C in 95% relative atmospheric humidity. Then 1.5 mL of culture medium was added to each well.[19]

Experimental Phases

To establish the natural growth curve of cells on titanium disks, osteoblasts, MC3T3-E1, 4×10^4 cells in passage 6 were cultivated in a complete culture medium for 21 days. Culture medium was changed every 3 days. A cell-viability test using MTT assay was performed every 3 days after cell seeding to establish the natural growth curve of cells (Fig 1a).

When the growth curve of cells on titanium disks was plotted, stages of cell growth were delinated into 3 experimental phases: static, log, and plateau. The static phase began 24 hours after cell seeding, the log phase on culture day 5, and the plateau phase on culture day 12 (Fig 1b).

Drug Treatment

In each experimental phase, cells were incubated in serum-free culture medium for 7 days. On the first day of each phase, culture medium supplemented with serum was removed and cells on disks were washed with phosphate-buffered saline (PBS) and cells were incubated in serum-free culture medium for 24 hours. The medium was then treated with indomethacin, celecoxib, or only dimethyl sulfoxide (DMSO) according to groups of the study. Subsequently, investigations were performed on treatment days 1, 3, and 5 (24, 72, and 120 hours in treated medium, respectively) (Fig 1b).

Preparation of Stock Solutions

The nonspecific COX-1 and COX-2 inhibitor NSAID used in this study was indomethacin (Sigma, St Louis, MO, USA), and the specific COX-2 inhibitor was celecoxib (Pfizer, USA). Stock solution was made in 100%

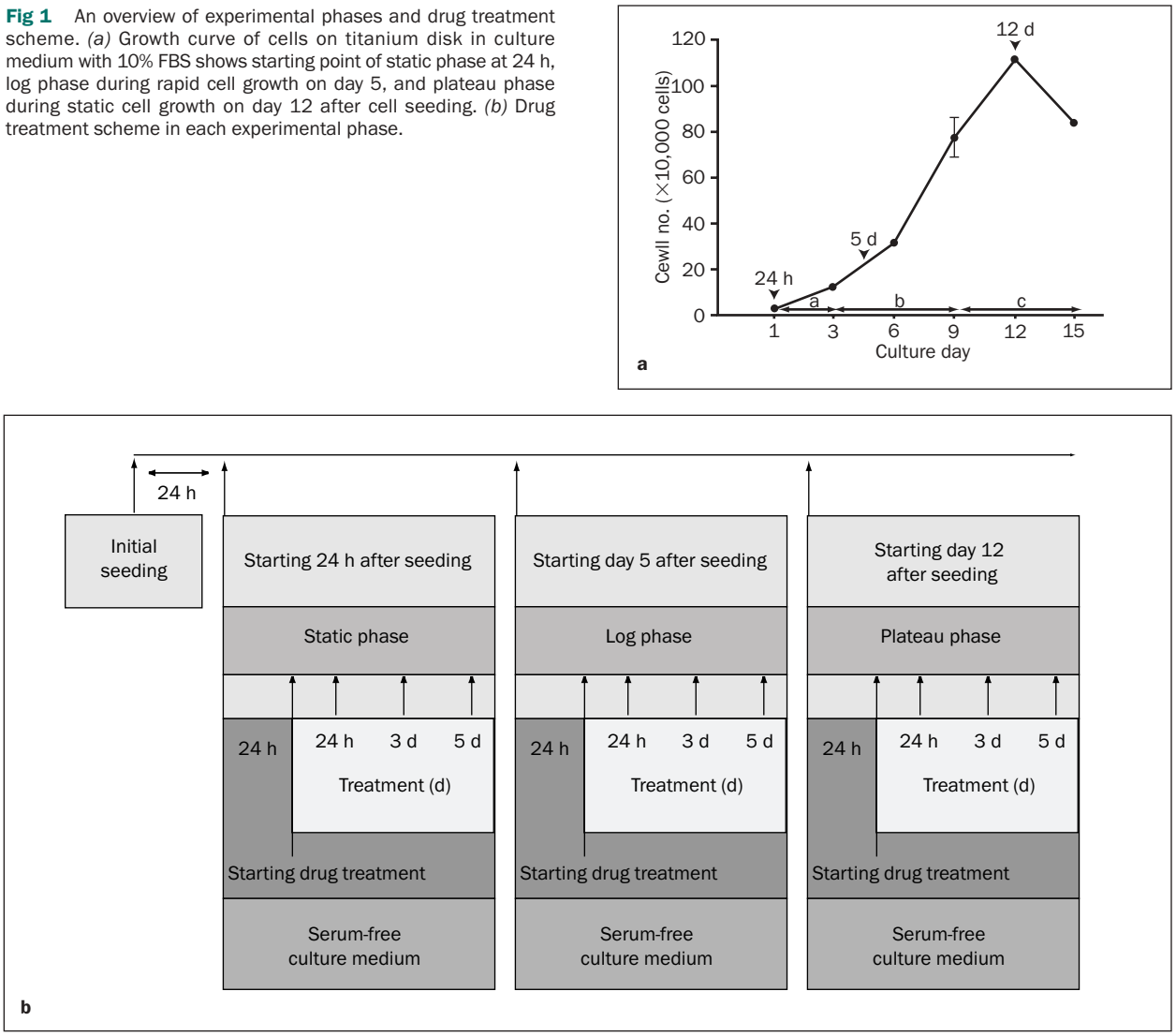

Fig 1 An overview of experimental phases and drug treatment scheme. *(a)* Growth curve of cells on titanium disk in culture medium with 10% FBS shows starting point of static phase at 24 h, log phase during rapid cell growth on day 5, and plateau phase during static cell growth on day 12 after cell seeding. *(b)* Drug treatment scheme in each experimental phase.

DMSO (Sigma) and stored at –20°C. The drug was diluted with culture media without fetal bovine serum (FBS) just prior to use. In the control group, E, 10 µL DMSO was added in 10 mL of culture medium. The concentration of DMSO for all treatments was 0.1%.[20]

Investigated Parameters and Investigation Schemes

Attachment of cells on titanium surfaces, cell growth, osteoblastic differentiation, and secretion of PGE_2 in culture medium were investigated. Attachment and morphology of cells on titanium disks were monitored under a scanning electron microscope (SEM) and confocal laser–scanning microscope (CLSM). Cell viability was determined using an MTT assay to demonstrate growth of cells. Osteoblastic differentiation was examined by measuring levels of ALP activity in cells and extracellular matrix and levels of osteocalcin in culture medium. Levels of PGE_2 in culture medium were measured to demonstrate inhibitory effects of NSAIDs on the function of cyclooxygenase enzymes on PGE_2 synthesis.

Characterizing Attachment and Growth of Cells on Titanium Surface (SEM)

On culture day 7, cells on titanium disks cultivated in culture medium with and without serum were washed twice with PBS and fixed with 2.5% glutaraldehyde in PBS and 1% osmium tetroxide. They were critically point dried and subsequently sputtered and coated with gold-palladium. The samples were observed using SEM (JEOL, JSM-5800LV, Japan). Fields of analysis were examined at both high and low magnifications to give a qualitative confirmation of morphology and attachment of cells onto the disks.[21]

Confocal Laser Scaning Microscope (CLSM)

Cell viability and distribution of cells on each disk of groups A, C, and E on treatment day 5 were examined using CLSM. On treatment day 5, the disks were washed twice with PBS and then incubated in 1.5 mL of 2 μg/mL fluorescein diacetate (FDA) in serum-free medium for 15 minutes in a 5% CO_2 incubator at 37°C in 95% relative atmospheric humidity. The disks were rinsed twice with PBS and examined within 30 minutes under CLSM (Olympus FV300, Japan).[22]

Cell Viability Assay

Cell viability was determined using a CellTiter 96 nonradioactive cell-proliferation assay (Promega, Madison, WI, USA). The measurement procedure was performed according to the manufacturer's instructions. Cells were incubated in culture medium containing 10% CellTiter 96 solution for 2 hours in 5% CO_2 at 37°C and then an equal volume of solubilization solution/stop-mix was added into each well and left in the plate to stand overnight in the incubator. The incubated medium was read at 562 nm absorbance in triplicate using a microplate reader (Biotrak II microplate reader, UK). Cell numbers were calculated from a standard curve of cell numbers and optical density.[23]

Measurement of Protein Concentration

An analysis of protein concentration in cell lysate solution was performed according to the Lowry assay using aliquots of cell lysate. An analysis was performed according to the manufacturer's instructions (Bio-Rad, DC Protein Assay Instruction Manual). Levels of protein in the cell lysate solutions were measured by monitoring light absorbance at 620 nm (Biotrak II microplate reader). Concentrations of protein were calculated from a standard curve of a series of protein standards. A total cellular protein analysis was performed on the same samples as alkaline phosphatase (ALP) activity and osteocalcin and PGE_2 measurements.

Measurement of ALP Activity

The assay aimed to measure ALP activity of protein extract solution obtained from detergent cell solubilization using 1% Triton X-100 in PBS. Cell lysis solutions were thawed on ice. Four hundred microliters of lysis buffer containing 2 mg p-Nitrophenyl phosphate in 1 mL of 1.5 M of 2-amino-2-methyl-1-propanolol were pipetted into each tube containing 100 μL of cell lysate solution. The solution was thoroughly mixed and incubated at 37°C for 1 hour. The reaction was stopped by adding 400 μL of 0.05 N NaOH and 100 μL of distilled water into each tube. Levels of p-Nitrophenol in the solutions were measured by monitoring light absorbance at 405 nm (Biotrak II microplate reader). The levels of intensity of yellow color are directly proportional to concentrations of p-Nitrophenol in standard solutions. The specific activity of ALP was neutralized with an amount of protein in the cell lysis solution and reported as units/mg of protein.

Measurement of Osteocalcin in Culture Medium

Levels of osteocalcin in the culture medium of cells undergoing ALP activity analysis were measured using a mouse osteocalcin EIA kit (BT-490, Biomedical Technologies, Stoughton, MA, USA). An analysis was performed according to the manufacturer's instruction. The mixture was measured absorbance at 450 nm using a microplate reader (Biotrak II microplate reader). Concentrations of osteocalcin were extrapolated from a standard curve of serial dilution of highly purified mouse osteocalcin (Mouse Osteocalcin EIA Kit Instruction Manual, Biomedical Technologies). The concentrations of osteocalcin were neutralized with an amount of protein in cell lysis solution and reported as a ng/mg of protein.

Measurement of PGE_2 in Culture Medium

Levels of PGE_2 in culture medium of cells undergoing ALP activity analysis were measured using a prostaglandin E2 high-sensitivity immunoassay (DE2100, R&D Systems, Minneapolis, MN, USA). An analysis was performed according to the manufacturer's instruction. Light absorbance was measured at 405 nm with wavelength correction set at 562 nm using a microplate reader (BiotrakTM II). Concentrations of PGE_2 were extrapolated from a standard curve of serial dilution of highly purified PGE_2. The concentrations of PGE_2 were neutralized with an amount of protein in cell the lysis solution and reported as a ng/mg of protein.

Statistical Analysis

The results of cell vitality and ALP assays are presented as the mean ± standard deviation (SD) of 2 separate experiments performed in triplicate (n = 6). The results of osteocalcin and PGE_2 assays are presented as the mean ± SD of an experiment, performed in triplicate (n = 3). Differences among groups were analyzed by analysis of variance (ANOVA) followed by the Tukey HD and Dunnette T3 tests. Time-effects level of each experiment within subject were analyzed by repeated measure ANOVA.

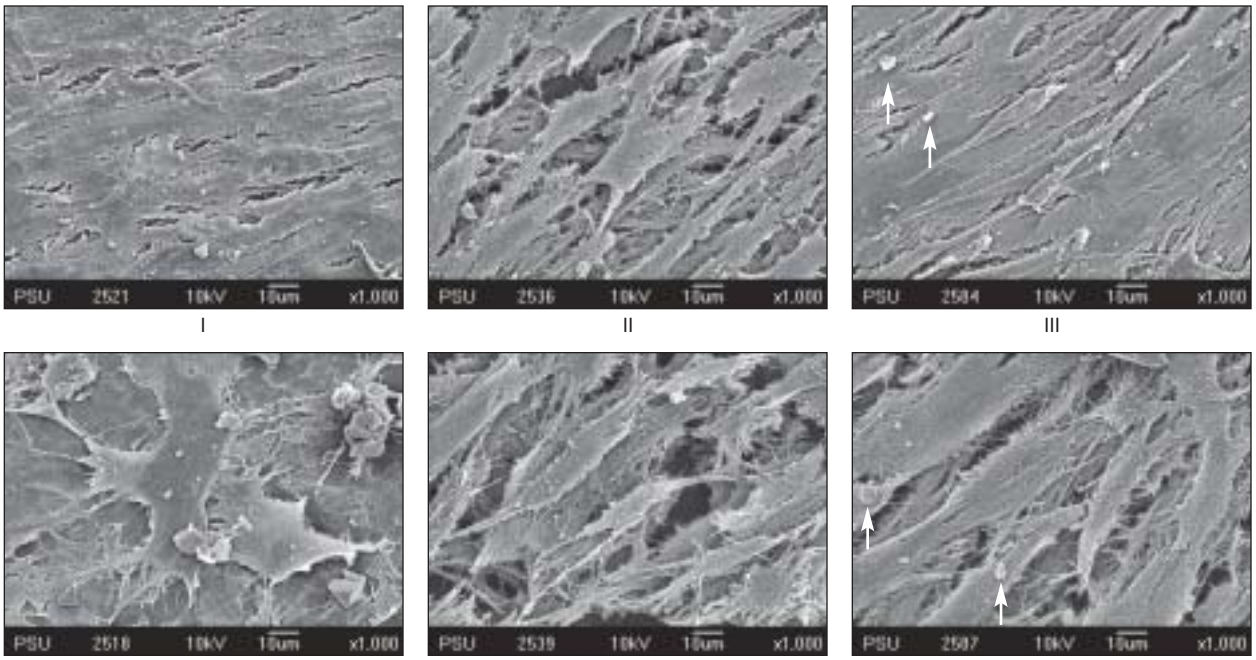

Fig 2 SEM images of MC3T3-E1 on surface of titanium disks cultured for 7 days (on drug treatment day 5) in culture medium *(a)* with and *(b)* without serum. (I) static, (II) log, and (III) plateau phases. Arrows indicate extracellular matrix (original magnification × 1,000).

RESULTS

Attachment and Morphology of Cells on Titanium Disks

Cells were able to grow and proliferate on the surface of titanium disks both in culture medium supplemented with and without serum. Osteoblasts were polygonal with numerous filopodia extending in multiple directions. Cells sprout their cytoplasmic processes on the titanium surface to create intercellular contact and grow in multiple layers (Fig 2, a and b). A deposit of extracellular matrix on cell surfaces can be observed in log and plateau phases (Fig 2, II and III).

Cell growth and attachment in serum-free culture medium, group E, was limited particularly in the static phase (Fig 2, I). Numbers of cells and spreading of cell cytoplasm and filopodia were markedly greater in culture medium with serum than serum-free culture medium (Fig 2, a and b).

CLSM Images of Attachment and Distribution of Cells on Titanium Disks

Confocal fluorescence micrographs showed that cells in groups A, C, and E on treatment day 5 (or culture day 7 in serum-free conditions) were vital. Cells attached well and sprouted their cytoplasmic process on the titanium surface to create intercellular contact covering approximately 80% to 90% of the surface of titanium disks. Effects of 0.1 μM indomethacin and 3 μM celecoxib on viability, attachment, and morphology of cells could not be differentiated from confocal fluorescence micrographs (Fig 3).

Effects of Drug Treatment on Cell Viability

It is clearly demonstrated that the numbers of cells consistently increased from static to plateau phases. The highest numbers of cells were found in plateau followed by log and static phases (Figs 4 to 6).

In the static phase, in comparison to growth of cells in the control group E (culture medium without drug treatment), a marked suppression of cell growth was found in groups A to D on treatment days 3 and 5. On treatment day 1, growth of cells in all groups, did not differ significantly (ANOVA, $F = 1.013$, df 4,25, $P = .49$). Growth of cells among experimental groups, groups A to D, were not different throughout the investigation time but were significantly different from group E (control group) on days 3 and 5 (ANOVA day 3, $F = 6.461$, df 4,25, $P = .01$) (Fig 4).

In the log phase, the lowest level of cell growth was found in group A: 0.1 μM indomethacin. On treatment day 1, cell numbers in group A were significantly lower than group E (control) and groups B and C of 1.5 μM and 3.0 μM celecoxib (ANOVA, $F = 6.890$, $df = 4,25$, $P = .001$), respectively, but not signifi-

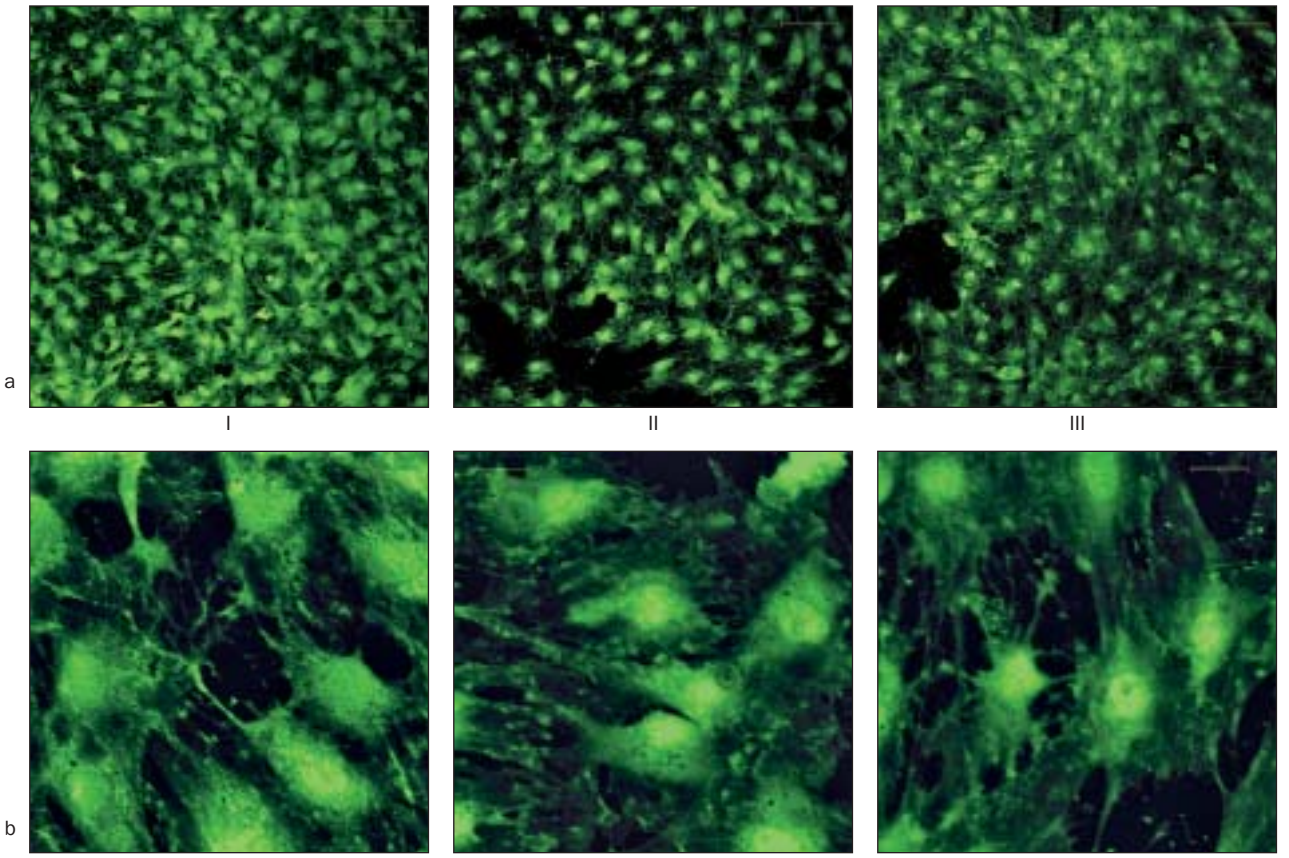

Fig 3 CLSM images of MC3T3-E1 on surface of titanium disks cultured for 7 days (on drug treatment day 5). (I) group E: the control group without drug treatment; (II) group A: 0.1 μM indomethacin, and (III) group C: 3.0 μM celecoxib. *(a)* Original magnification × 200, *(b)* original magnification × 1,000.

cantly lower than group D: 9.0 μM celecoxib. On treatment day 3, a suppression of cell growth was found in groups treated with indomethacin and celecoxib, groups A through D. Numbers of cells in group A were significantly lower than in group E, the control (Tukey HSD, MD = -4.064, $P = .003$), and tended to be lower than in groups B through D ($P > .05$). On treatment day 5, a significant decrease in numbers of cells was found only in group A. Numbers of cells in group A were significantly lower than in groups C and E (ANOVA, $F = 7.190$, $df = 4,25$, $P = .001$) (Fig 5).

In the plateau phase, different levels of cell growth among all groups of study were not found (repeated measure among subjects, $F = 0.004$, $df = 4,25$, $P = .997$). Growth of cells was stable during treatment days 1 through 5 (repeated measure within subject, $F = 0.279$, $df = 4,25$, $P = .889$) (Fig 6).

Effects of Drug Treatment on Alkaline Phosphatase Activity

Effects of drug treatment with celecoxib on ALP activity in dose- and time-dependent measures were inconsistent. A significant decrease of ALP activity in comparison to the control group E, culture medium without drug treatment (3.749 ± 0.601 unit/mg), was found in only group C, being 3.0 μM celecoxib in the static phase on treatment day 5 (1.769 ± 0.314 unit/mg) (Dunnett T3, MD = 1.979, $P = .001$) (Fig 7). In the log phase, levels of the activity of all groups were not significantly different (repeated measure between subjects, $F = 0.674$, $df = 4,25$, $P = .617$) (Fig 8).

Stimulating effects of celecoxib on ALP activity was found in the plateau phase on treatment days 1 and 3. On treatment day 1, the ALP activities of groups A and group B were significantly higher than of control group E (Dunnett T3, group A: MD = 1.671, $P = .023$; group C: MD = 0.831 ± 0.214, $P = .039$) (Fig 9).

On treatment day 3, in comparison to group E, a stimulatory effect was found in groups A and D (Mann-Whitney test, group A: $P = .009$; group D: $P = .001$). The level in group B (1.795 ± 0.513 unit/mg) was significantly lower than in groups A and D.

On treatment day 5, the ALP activity of group D, 9.0 μM celecoxib, was higher than other groups, but there was no statistical difference (repeated measure between subjects, $F = 0.674$, $df = 4,25$, $P = .617$) (Fig 9).

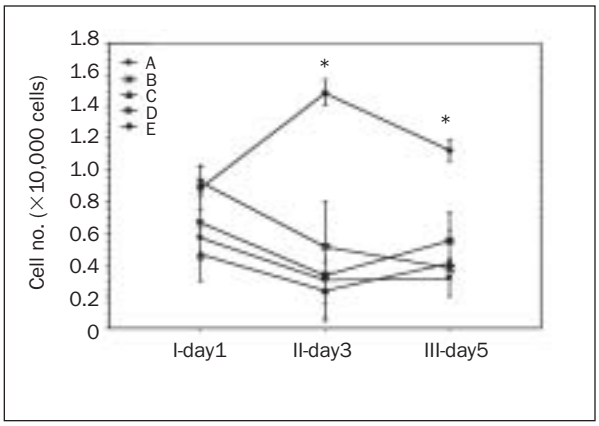

Fig 4 Growth of cells in the static phase on treatment days 1, 3, and 5. *$P < .05$; significant difference compared with all experimental groups, groups A to D (mean ± SE of 3 determinations, each in duplicate, n = 6).

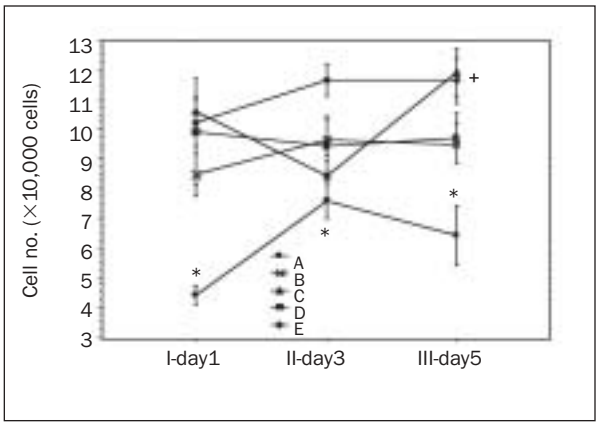

Fig 5 Growth of cells in the log phase on treatment days 1, 3, and 5. *$P < .05$; significant difference compared with the control, group E. +$P < .05$; significant difference compared with group A: 0.1 μM indomethacin (mean ± SE of 3 determinations, each in duplicate, n = 6).

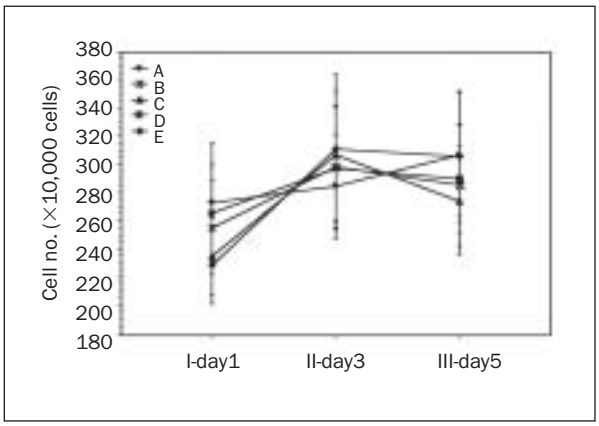

Fig 6 Growth of cells during drug treatment in the plateau phase. Numbers of cells among groups were not significantly different (mean ± SE of 3 determinations, each in duplicate, n = 6).

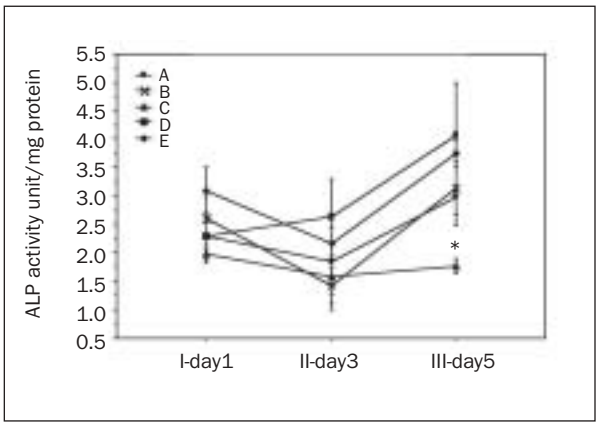

Fig 7 Alkaline phosphatase (ALP) activity of cells on titanium disks in the static phase on treatment days 1, 3, and 5. *$P < .05$; significant difference compared with the control, group E (mean ± SE of 3 determinations, each in duplicate, n = 6).

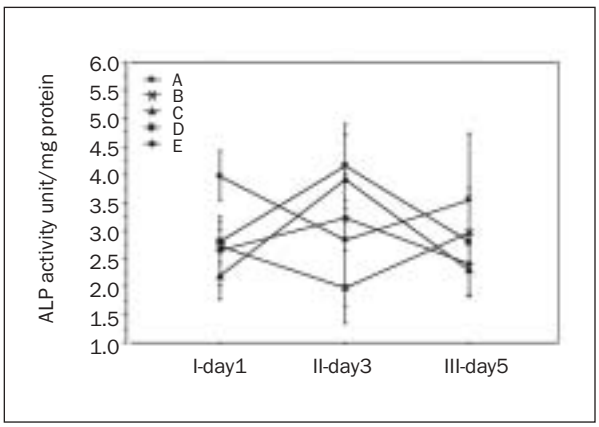

Fig 8 Alkaline phosphatase (ALP) activity of cells on titanium disks in the log phase on treatment days 1, 3, and 5. The activity of cells among groups was not significantly different (mean ± SE of 3 determinations, each in duplicate, n = 6)

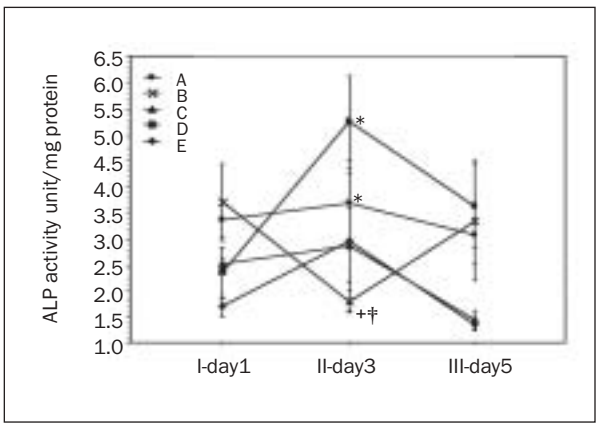

Fig 9 Alkaline phosphatase (ALP) activity of MC3T3-E1 on titanium disks during drug treatment in the plateau phase. *$P < .05$; significant difference compared with the control, group E. +$P < .05$: significant difference compared with group A: 0.1 μM indomethacin. †$P < .05$; significant difference compared with group D: 9.0 μM celecoxib (mean ± SE of 3 determinations, each in duplicate, n = 6).

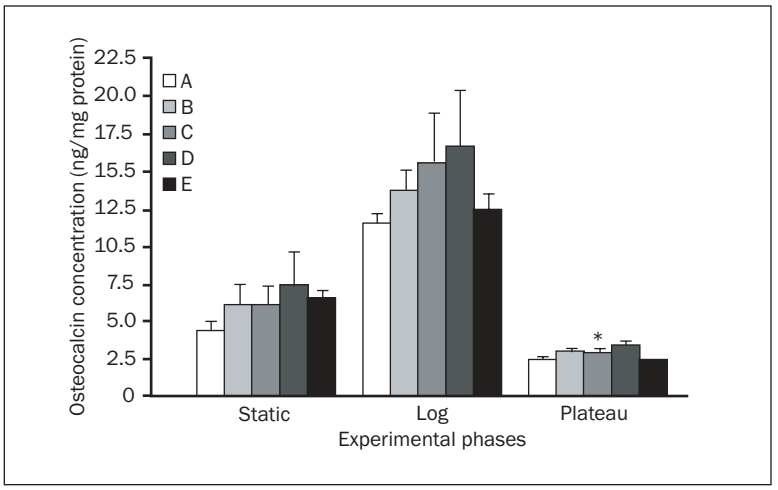

Fig 10 Levels of osteocalcin in culture medium on treatment day 5 in experimental static, log, and plateau phases (mean ± SE, n = 3). *$P < .05$; all groups in the plateau phase exhibited significant differences compared with all groups in the static and log phases (mean ± SE, n = 3).

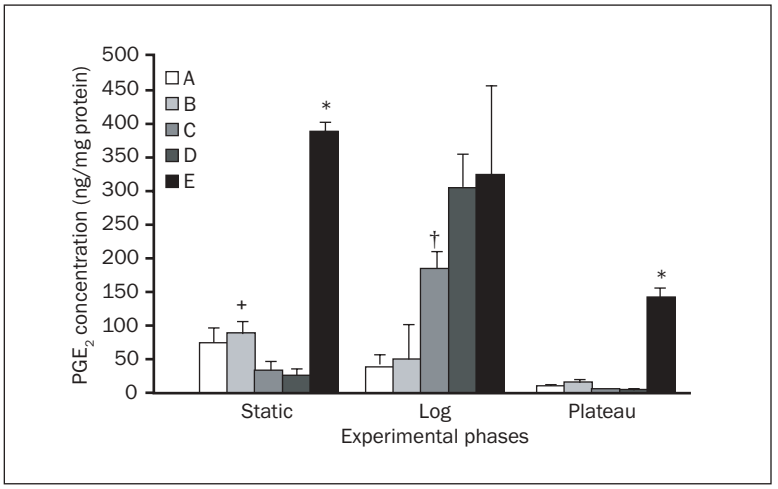

Fig 11 Levels of PGE_2 in culture medium on treatment day 5 in each experimental phase, static, log, and plateau phases. *$P < .05$; significant difference compared with all experimental groups (groups A to D). +$P < .05$; significant difference compared with group B in the plateau phase. †$P < .05$; significant difference compared with group C in the static phase (mean ± SE, n = 3).

Effects of Drug Treatment on Levels of Osteocalcin in Culture Medium

In every phase of the study, static, log, and plateau phases, levels of osteocalcin in all experimental groups, groups A to D, were not significantly different from group E (control). Levels of osteocalcin in the log phase (7.869 ± 2.789 ng/mg) tended to be higher than the levels in the static phase (3.672 ± 1.465 ng/mg), but they were not significantly different (Fig 10). The lowest level of osteocalcin was found in the plateau phase (1.806 ± 0.705 ng/mg), where the levels of osteocalcin of all groups in the plateau phase were significantly lower than the levels in the static and log phases (repeated measure, F = 173.951, $df = 1$, $P = .000$).

Effects of Drug Treatment on Levels of PGE_2 in Culture Medium

Levels of PGE_2 in experimental groups A-D were significantly lower than in group E, the control group ($P < .05$), in static (ANOVA, F = 44.085, $df = 4,10$, $P = .00$) and plateau phases (ANOVA, F = 120.728, $df = 4,10$, $P = .00$), while significant differences were not found in the log phase.

Levels of PGE_2 of groups A, D, and E were stable in all experimental phases, static, log and plateau phases (Fig 11). A significant change of PGE_2 levels was found in groups B and C. The level of PGE_2 for group B in the static phase was significantly higher than the plateau phase (*t* test, $t = 6.537$, $df = 2$, $P = .023$) but it was not significantly different from the log phase. For group C, the level in the log phase was significantly higher than the plateau phase (*t* test, t = 8.282, $df = 2$, $P = .014$) (Fig 11).

DISCUSSION

This study investigated the effects of a specific COX-2 inhibitor on growth and differentiation of osteoblasts on an acid-prickled titanium surface.

Influence of exposure time and stages of cell growth on the effects of specific COX-2 inhibitor NSAIDs were investigated and compared with a nonspecific COX-1 and COX-2 inhibitor NSAID (indomethacin in therapeutic doses). In the present study, despite a reduction of cell growth found in cell viability assay on treatment day 5 or culture day 7 in serum-free culture medium (Figs 4 to 6), a limiting cell adhesion and growth on a titanium surface found in serum-free culture medium on SEM image (Fig 2b) were overcome by accumulation of ECM and intercellular contact found in the log and plateau phases (Fig 2b, II and III). This might be because a lack of protein deposition on the titanium surface and essential growth factors in serum-free culture medium[24] were compensated for by the stimulatory effects of intercellular contact and autocrine functions of growth factors in extracellular matrix on the growth of osteoblasts.[25] This evidence implied that effects of external stimuli on growth and differentiation of cells could be influenced by stages of cell growth or density of cells growing on a titanium surface. CLSM images further emphasized that NSAID treatment did not affect attachment and morphology of cells on titanium disks (Fig 3).

The effects of specific COX-2 inhibitor NSAIDs on proliferation and differentiation of osteoblasts are inconsistent. The present study agreed with Evan and Butcher[14] that nonspecific and specific COX-2 inhibitor NSAIDs inhibited growth of osteoblasts but disagreed with Boyan and coworkers[15] that a specific COX-2 inhibitor did not have any effect on proliferation of osteoblasts on a smooth titanium surface. Regarding the effects on differentiation of cells, the current findings agreed with previous reports[15] that specific COX-2 inhibitor NSAID, celecoxib, did not interfere with the progress of osteoblastic differentiation through to mature stages (Fig 10). It was found that indomethacin and celecoxib did not clearly show their effect on ALP and osteocalcin levels (Figs 9 and 10). Dose-dependent effects of conventional and specific COX-2 inhibitor NSAIDs have been clearly demonstrated in most studies.[14,26] The inhibitory effect of indomethacin and celecoxib in dose- and time exposure–dependent measures found in the present study supported previous in vivo studies reporting inhibitory effects of specific COX-2 inhibitors, celecoxib and rofecoxib, on bone healing[27] and bone ingrowth into porous chrome-cobalt[28] and titanium implants[11,29] by inhibiting proliferation of cells in a dose- and time exposure–dependent manner.

The significant decrease of PGE_2 levels found in the present study (Fig 11) implied that indomethacin and celecoxib were able to inhibit the function of the cycloxygenase pathway by blocking synthesis of the cyclooxygenase enzymes and that the effects were influenced by stages of cell growth and doses of celecoxib (Fig 11). The finding agreed with Coetzee et al,[30] who reported that specific COX-2 inhibitors, NS-398 0.1 μM and indomethacin 1.0 μM, inhibited PGE_2 production of MC3T3-E1 and MG63.

However, changes of cell growth, ALP activity, and osteocalcin levels did not correspond with a decrease of PGE_2 synthesis found in each experimental phase. Thus, it was unlikely that the effects of NSAIDs on growth and differentiation of osteoblasts were the result of reduction of PGE_2 function. This assumption is supported by Cheng and coworkers[31] that the effect of celecoxib on the differentiation of osteoblasts on a smooth titanium surface is not via a reduction of PGE_2 production. This may be because osteoblastic differentiation is a complex multifactorial event and PGE_2 is not the only factor regulating osteoblastic differentiation.[32] The results led to a postulation that effects of specific COX-2 inhibitors on growth and differentiation of osteoblasts did not critically depend on a reduction of PGE_2 synthesis.

The present study demonstrated the influence of stages of cell growth on effects of celecoxib on growth and differentiation of osteoblasts by adding the drugs in culture medium at different stages of cell growth; the static, log, and plateau phases that are comparable to 3 stages of osteoblastic differentiation: proliferation, matrix formation, and mineralization.[33] A variation of effects of NSAIDs on growth and differentiation of osteoblasts in different drug treatment time and phases of study suggested that the effects of both nonspecific and specific COX-2 inhibitor NSAIDs on osteoblasts was influenced by drug exposure time and stages of osteoblastic differentiation (Figs 4 to 6).

Osteointegration involves a complex series of events and is tied to discrete phases of osteogenesis, including osteogenic differentiation, matrix production, mineralization, and remodeling.[34] Thus, growth and differentiation of osteoblasts are prime factors determining the success or failure of osteointegration. Additionally, osteoblatic differentiation is temporally regulated and is closely related to stages of cell growth.[35] Hence, the findings of the present study, that the inhibitory effect of celecoxib on cell growth was influenced by stages of cell growth and the inhibitory effect of indomethacin was greater than celecoxib, suggested that specific COX-2 inhibitors could interfere with osseointegration of dental implants and the effects of NSAIDs on different stages of osseointegration could be different. Effects of a specific COX-2 inhibitor, celecoxib, are expected to be lower than indomethacin or conventional NSAIDs.

In the current study, the concentrations of the treated indomethacin and celecoxib were comparable to their therapeutic plasma levels. The therapeutic doses of indomethacin is 50 to 200 mg or 284 ng/mL in plasma[36] and celecoxib is 100 to 200 mg/day for osteoarthritis, 200 to 400 mg/day for rheumatoid arthritis, and 200 mg/day for acute pain.[37] With a half life of 12 hours, plasma levels of therapeutic doses of 100, 400, and 800 mg/day are equivalent to 1.7, 3.1, and 9.2 μM, respectively.[37] Therefore, celecoxib in therapeutic doses of 100 to 400 mg/day could pose adverse effects on growth of osteoblasts on a titanium surface, particularly in stages of early and rapid cell growth, and the effects are expected to increase with the doses. The findings suggested that adverse effects of celecoxib tended to be lower than those of indomethacin (Figs 4 and 5).

CONCLUSION

The results of this study demonstrated that indomethacin and celecoxib in therapeutic doses were able to inhibit the growth of osteoblasts on smooth titanium surfaces in dose- and time-dependent manners and the effects were influenced by stages of cell growth. The findings suggested that celecoxib in therapeutic doses of 200 to 400 mg/day for more than 3 days could jeopardize osseointegration by inhibiting proliferation of cells in the proliferative stage of osseointegration, particularly in high doses and with longer exposure time.

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