รายงานวิจัยฉบับสมบูรณ์

จากเงินอุดหนุนการวิจัยจากเงินรายได้มหาวิทยาลัยสงขลานครินทร์

ระหว่างเดือน กรกฎาคม ปี พ.ศ. 2549 ถึง เดือน กรกฎาคม ปี พ.ศ. 2550

โครงการ

ผลของสารยับยั้งที่มีผลเฉพาะเจาะจงต่อการทำงานของเอนไซม์ไซโครออกซิเจเนส-2 ต่อการเจริญเติบโต และการพัฒนาของเซลล์สร้างกระดูกบนผิวของไททาเนียมที่ใช้ทำวัสดุเทียม

(Effects of specific Cox-2 inhibitor on growth and differentiation of osteoblasts on titanium surface of implant, an *in vitro* study)

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ABSTRACTS

Purpose: Specific COX-2 inhibitor NSAID, celecoxib, is widely received as an alternative analgesic to conventional NSAIDs in treating chronic and acute pain. The current study aimed to investigate effects of influence of exposure time and stages of cell growth on effects of specific COX-2 inhibitor NSAID on growth and differentiation of osteoblasts on smooth titanium surface. ***Materials and Methods:*** The study was categorized into 5 groups, Group A: 0.1 μM indomethcin; 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. Mouse calvarial cell line, MC3T3-E1, was seeded on acid prickled surface titanium disks (Straumann, Waldenburg, Switzerland). The investigations were performed on three different experimental phases basing on stages of cell growth, *static* (at 24 hours after seeding), *log* (on culture-day 5) and *plateau* (on culture-day 12) phases. In each experimental phase, cells on titanium disks were incubated in medium treated with drugs according to groups of study for 1, 3 and 5 days. ***Results:*** It was found that indomethacin and celecoxib in Groups A-D inhibited growth of cells on treatement days 3 and 5 in *static* phase and treatment day 3 in *log* phase and an inhibitory effect of indomethacin was greater than celecoxib. Effects on ALP activity and osteocaclin were not clearly demonstrated. Significant decrease of PGE_2 production was found in Groups A-D in *static* and *plateau*, but not in *log* phases. ***Conclusions:*** Specific COX-2 inhibitor NSAID, celecoxib, was able to inhibit growth of osteoblasts on titanium surface and the effects were influenced by exposure time and stages of cell growth. Undertaking of specific COX-2 inhibitor might cause deteriorate effects on osteointegration of dental implant by interfering with osteoblastic cell growth in proliferative stage.

KEYWORDS

Specific COX-2 inhibitor NSAIDs, Osteoblasts, Titanium Surface, Growth and differentiation

INTRODUCTION

Non-specific COX-1 and COX-2 inhibitor NSAIDs decrease inflammation by inhibiting catalyst function of cyclooxygenase 1 and 2 (COX-1 and COX-2) resulting in reductions of prostaglandin and thromboxane A2 synthesis[1,2]. Specific COX-2 inhibitor NSAIDs, celecoxib, is developed to avoid adverse effects from inhibition of the constitutive function of COX-1 and well received as an analgesic drug for management of chronic and acute pain[3,4]. Because COX-2 is an inducible enzyme produced in responding to stress and trauma, this evidence suggests an important role of COX-2 in the early stage of wound healing and repair[3]. Thus specific COX-2 inhibitor NSAIDs do not inhibit only the inflammatory process, but also a production of prostaglandins which are necessary for bone healing and metabolism[5,6].

Evidences from animal studies demonstrates that COX-2 inhibitors inhibit bone cell proliferation and delay fracture healing in dose and time dependent manners[7,8]. Roles of COX-2 in osteogenesis and bone metabolism are further emphasized by Zhang and co-workers[9] that 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 inhibitor *in vivo* studies are supported by *in vitro* studies that specific COX-2 inhibitors and conventional NSAIDs, inhibit proliferation of human osteoblasts on the plastic surface of cell culture plate[10] and decrease differentiation of osteoblasts on rough titanium surfaces [11].

Although adverse effects of specific COX-2 inhibitor NSAIDs on growth of osteoblats, bone healing and osteogenesis are reported, there is insufficient evidence to define effects of specific COX-2 inhibitors NSAIDs on osteointegration of implant prosthesis, particularly in the use of high doses and/or extended period of time such as

in osteoarthritis and rheumatoid arthritis cases in which celecoxib must be taken for at least 2 weeks[12,13].

Osteoarthritis is one of the most common indication for COX-2 therapy and osteoarthritis patients have average age of more than 60 year old[9,14,15]. This group of patients is people who most need dental implant for their quality of life[16] and bone metabolism is one of many important factors contributing to success of implants[17]. Thus, further studies are required to demonstrate influence of dose, timing and duration of specific COX-2 inhibitor administration on osteogenesis and osteointegration.

In the presence study, a study of growth and differentiation of MC3T3E-1 cell line on a titanium surface *in vitro* was selected as a study model to investigate effects of specific COX-2 inhibitor NSIADs on growth and differentiation of osteoblasts on the surface of titanium implant[18,19,20,21]. MC3T3E-1 cell line is widely used in biocompatibility testing and studying effects of matrix interaction and external stimuli on growth and differentiation of osteoblasts[22,23,24]. The cell line is well documented for its capacities to proliferate and differentiate while expressing osteoblastic phenotypes including ALP activity, osteocalcin and calcium production[25,26].

For the current study, the authors intended to examine factors influencing adverse effects of specific COX-2 inhibitor NSAIDs on osteointegration of dental implant by investigating dose and exposure time dependent effects of specific COX-2 inhibitor on growth and differentiation of osteoblasts on smooth titanium surface in different stages of cell growth *in vitro*. It is 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

the subsequent of inhibitory effect of cyclooxygenase enzymes on the production of prostaglandin E_2 (PGE_2). .

MATERIALS AND METHODS

GROUPS OF STUDY

The study was categorized into five groups according to treated drugs in 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 acid pricked surface, 15 mm in diameter with 1 mm thickness were kindly provided by Straumann (Straumann, Waldenburg, Switzerland). Disks were prepared for cell seeding according to Lohmann and co-workers[27]. Procedures in brief, prior to cell seeding, disks were sonicated (Cavitator®ME11, Mettler Electronics Corp), rinsed in double distilled water, neutralized in 5% sodium bicarbonate and rinsed in deionized water. After that disks were air dried and sterilized by autoclaving. Disks were placed in 24-well culture plate one disk in one well for cell culture on titanium surface.

MC3T3-E1 CELL CULTURE

MC3T3-E1 mouse cell line was obtained from ATCC (USA). The cell line throughout cell culture period was cultivated in alpha-MEM medium, 10% FBS, 1% Penicillin/streptomycin, 0.5% Fungizone and 50 μg/ml ascorbic acid. Culture medium was changed every 2 days[28]. Cells were cultivated into the fourth – sixth passages. When growth of cells reached 80% confluence, cells were tripsinized and

seeded on titanium disks, $4x10^4$ 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 were added into each well[29].

EXPERIMENTAL PHASES

The study was categorized into 3 experimental phases, (I) *static*, (II) *log* and (III) *plateau* phases according to stages of cell growth on titanium disks. *Static* phase was started at 24 hours after cell seeding, *log* phase on culture-day 5 and *plateau* phases on culture-day 12 (Fig 1).

DRUG TREATMENT

In each experimental phase, cells were incubated in serum free culture medium for 7 days. On the starting date of each phase, culture medium supplemented with serum was removed and cell on disks were washed with PBS, and then cells were incubated in serum free culture medium for 24 hours. After that, the medium was treated with indomethacin, celecoxib or only DMSO according to groups of the study. Subsequently, investigations were performed on treatment-days 1, 3 and 5 (24, 72 and 12o hours in treated medium, respectively) (Fig 1).

PREPARATION OF STOCK SOLUTIONS OF TREATED MEDICATIONS

The non-specific COX-1 and COX-2 inhibitor NSAIDs used in this study was indomethacin (Sigma, USA) and specific COX-2 inhibitor was Celecoxib (Pflizer, USA). Stock solution was made in 100% DMSO (Sigma, St Luis, MO) and stored at -20°C. Drug was diluted with culture media without FBS immediately just before use. In the control group, Group E, 10 µl DMSO was added in 10 ml of culture medium. The concentration of DMSO for all treatments was 0.1%[30].

INVESTIGATED PARAMETERS AND INVESTIGATION SCHEMES

Attachment of cells on titanium surface, 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 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 function of cyclooxygenase enzymes on PGE_2 synthesis.

CHARACTERIZING ATTACHMENT AND GROWTH OF CELLS ON TITANIUM SURFACE (SEM)

On culture-day 7 of cells on titanium disks cultivated in culture medium with and without serum, culture medium was removed and the disks were washed twice with PBS to remove unattached cells on the surfaces. Then the disks were fixed with 2.5% glutaraldehyde in PBS and 1% osmium tetroxide then critically point dried and subsequently sputtered and coated with gold-palladium. The samples were observed using SEM (JEOL, JSM-5800LV model, Japan). Fields of analysis were examined at both high and low magnifications to give a qualitative confirmation of morphology and attachment of cells on to the disks[31].

CONFOCAL LASER SCANING MICROSCOPE (CLSM)

Cell viability and distribution of cells on each disk of Groups A, C and E on treatment day 5 was examined using CLSM. On treatment-day 5, culture medium was removed and the disks were washed twice with PBS. Then 1.5 ml of fluorescein

diacetate (FDA), 2 μg/ml in serum free medium was added into each well and incubate in 5% CO_2 incubator at 37°C in 95% relative atmosphere humidity for 15 minutes. After that the disks were rinsed twice with PBS and examined within 30 minutes under CLSM (Olympus, FV300 model, Japan). Fields of analysis were examined at both high and low magnifications to give a qualitative confirmation of attachment and distribution of cells on the disks[32].

CELL VIABILITY ASSAY

Cell viability was determined using CellTiter 96□ Non-Radioactive Cell proliferation Assay (Promega, USA). Measurement procedure was performed according to manufacturer's instruction. In brief, 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 the plate to stand overnight in the incubator. Then the incubated medium was read at 562 nm absorbance in triplicate using microplate reader (BiotrakTM II microplate reader, UK). Cell number was extrapolated from standard curve of cell numbers and optical density [33].

MEASUREMENT OF PROTEIN CONCENTRATION

An analysis of protein concentration in cell lysate solution was performed according to Lowry assay using aliquots of cell lysate. An anlysis was performed according to manufacturer's instruction (Bio-Rad, DC Protein Assay Instruction Manual). Levels of protein in the cell lysate solutions were measured by monitoring light absorbance at 620 nm (BiotrakTM II microplate reader, UK). Concentrations of protein were extrapolated from standard curve of series of protein standard (Bio-Rad, DC Protein Assay Instruction Manual).

MEASUREMENT OF ALP ACTIVITY

The assay is aimed to measure alkaline phosphatase activity of protein extract solution obtained from detergent cell solubilization using 1% Triton X-100 in PBS. A procedure in brief: 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. Then 400 µl of 0.05 N NaOH and 100 µl of distilled water were added into each tube to stop the reaction. Levels of p-Nitrophenol in the solutions were measured by monitoring light absorbance at 405 nm (BiotrakTM II microplate reader, UK). The levels of intensity of yellow color are directly proportional to concentrations of p-Nitrophenol in standard solutions. The specific activity of ALP was calculated as units/mg protein.

MEASURMENT OF OSTEOCALCIN IN CULTURE MEDIUM

Levels of osteocalcin in culture medium of cells undergone ALP activity analysis was measured using Mouse osteocalcin EIA kit (BT-490, Biomedical Technologies Inc, USA). An analysis was performed according to manufacturer's instruction. The mixture was measured absorbance at 450 nm using microplate reader (BiotrakTM II microplate reader, UK). Concentrations of ostreocalcin were extrapolated from standard curve of serial dilution of highly purified mouse osteocalcin and reported as ng/mg protein (Mouse Osteocalcin EIA Kit Instruction Manual, Biomedical Technologies Inc.).

MEASURMENT OF PGE_2 IN CULTURE MEDIUM

Levels of Prostaglandin E2 in culture medium of cells undergone ALP activity analysis was measured using Prostaglandin E2 High Sensitivity Immunoassay (DE2100, R&D Systems, Inc., USA). An analysis was performed according to manufacturer's instruction. A light absorbance was measured at 405 nm with wavelength correction set at 562 nm using microplate reader (BiotrakTM II microplate reader, UK) (Prostaglandin E2 High Sensitivity Immunoassay Instruction Manual).

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 ± standard deviation (SD) of an experiment, performed in triplicate (n=3). Statistical analysis was performed by analysis of variance (ANOVA) followed by the Tukey HD tests and Dunnette T3.

RESULTS

ATTACHMENT AND MORPHOLOGY OF CELLS ON TITANIUM DISKS

Cells were able to grow and proliferate on surface of titanium disks both in culture medium supplemented with and without serum. Osteoblasts were polygonal in shape with numeral filopodia extending in multiple directions. Cells sprout their cytoplasmic processes on titanium surface to create intercellular contact and grow in multilayer. A deposition of extracellular matrix on cell surface can be observed in *log* and *plateau* phases (Fig 2).

Cell growth and attachment in serum free culture medium, Group E, was limited particularly in *static* phase. Numbers of cells and spreading of cell cytoplasm and filopodias were markedly greater in culture medium with serum than serum free culture medium (Fig 2).

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 condition) were vital. Cells attached well and sprouted their cytoplasmic process on titanium surface to create intercellular contact covering approximately 80-90% of surface of titanium disks. A high cell density was found in the central region of the 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 TREATED DRUGS ON CELL VIABILITY

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

In ***static*** **phase,** in comparison to growth of cells in a control Group E: culture medium without drug treatment, a marked suppression of cell growth was found in Groups A – D on treatment days 3 and 5. On treatment-day 1, growth of cells in all groups, Groups A-E, were not significantly different (ANOVA, $F=1.013$, df 4, 25 $p=0.49$). Growth of cells among experimental groups, Groups A-D, were not different throughout the investigation time but significantly different from Group E (Control group) on days 3 and 5 (ANOVA day 3, $F=6.461$, df 4,25, $p=0.01$) (Fig 4).

In ***log*** **phase**, the lowest level of cell growth was found in Group A: 0.1 μM indomethacin. On treatment-day 1, numbers of cells in Group A was markedly and significantly lower than Group E: a control group and Groups B and C of 1.5 μM and 3.0 μM celecoxib (ANOVA, F=6.890, df=4, 25, p=0.001) but not Group D: 9.0 μM celecoxib. On treatment-day 3, a suppression of cell growth was found in groups treated with indomethacine and celecoxib, Groups A-D. Numbers of cells in Group A were significantly lower than Groups E, control (Tukey HSD, MD=-4.064, p=0.003) and tended to be lower than Groups B-D (p>0.05). On treatment-day 5, a significant decrease of numbers of cells was found in Group A only. Numbers of cells in Group A was significantly lower than Groups C and E (ANOVA, F=7.190, df=4, 25, p=0.001) (Fig 5).

In ***plateau*** **phase**, different levels of cells growth among groups of study, Groups A-E, was not found (Repeated measure between subject, F=0.004, df=4.25, p=0.997). Growth of cells were stable during treatment-days 1-5 (Repeated measure within subject, F=0.279, df=4, 25, p=0.889) (Fig 6).

EFFECTS OF TREATED DRUGS ON ALKALINE PHOSPHATASE ACTIVITY

Effects of treated drugs on ALP activity of celecoxib in dose and time dependent manners were inconsistent. Significant decreased of ALP activity in comparison to control Group E: culture medium without treated drug (3.749±0.601 unit/mg) was only found in Group C: 3.0 μM celecoxib in *static* phase on treatment-day 5 (1.769±0.314 unit/mg) (Dunnett T3, MD=1.979, p=0.001) (Fig 7). In *log* phase, levels of the activity of all groups were not significantly different (Repeated measure between subjects, F=0.674, df=4, 25, P=0.617) (Fig 8).

Stimulating effects of celecoxib on ALP activity was found in *plateau* phase on treatment-days, 1 and 3. On treatment-day 1, the ALP activities of Group A: 0.1 μM indomethacin (3.371±0.836 unit/mg) and Group B: 1.5 μM celecoxib (2.531±0.247 unit/mg) were significantly higher than a control Group E (1.701±0.461 unit/mg) (Dunnett T3, Group A: MD=1.671, p=0.023; Group C: MD=0.831±0.214, p=0.039).

On treatment-day 3, in comparison to a control group, Group E (2.948±2.253 unit/mg), a stimulatory effect was found in Groups A (3.677±2.016 unit/mg) and D (2.522±0.892 unit/mg) (Mann-Whitney test, Group A: p=0.009; P=0.001). The level in Group B (1.795±0.513 unit/mg) was significantly lower than Groups A and D (Group A: p=0.009; Group D: p=0.001).

On treatment-day 5, the ALP activity of Group D: 9.0 μM celecoxib was higher than other groups but there was no statistically different (Repeated measure between subjects, F=0.674, df=4 25, P=0.617) (Fig 9).

EFFECTS OF TREATED DRUGS 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-D, were not significantly different from Group E: a control group of cell culture on titanium disks without drug treatment. 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 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=0.000).

EFFECTS OF TREATED DRUG ON LEVELS OF PGE_2 IN CULTURE MEDIUM

Levels of PGE_2 of experimental groups, Groups A-D, were significantly lower than Group E: control group ($p<0.05$) in *static* (ANOVA, F=44.085, df=4,10, p=0.00) and *plateau* phases (ANOVA, F=120.728, df=4,10, p=0.00), while the significant differences were not found in *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 of Group B in *static* phase was significantly higher than *plateau* phase (T-test, t=6.537, df=2, P=0.023) but it was not significantly different from *log* phase. For Group C, the level in *log* phase was significantly higher than *plateau* phase (T-test, t=8.282, df=2, P=0.014) (Fig 11).

DISCUSSION

The study was conducted to investigate effects of specific COX-2 inhibitor on growth and differentiation of osteoblasts on acid prickled titanium surface. Influence of an exposure time and stages of cell growth on effects of specific COX-2 inhibitor NSAIDs were investigated and compared with non-specific 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 (Fig 4-6), growth of MC3T3-E1 cell line on titanium surface was similar to previous reports[34,35,36] (Fig 2) and SEM images presented that a limiting cell adhesion and growth on titanium surface found in serum free culture medium (Fig 2B-I) was overcome by accumulation of ECM and intercellular contact found in *log* and *plateau* phases (Fig 2B-J and II). This might be because a lack of protein deposition on titanium surface and essential growth factors in serum free

culture medium[37] were compensated by stimulatory effects of intercellular contact and autocrine functions of growth factors in extracellular matrix on growth of osteoblasts[28]. These evidences 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 treated NSAIDs did not affect attachment and morphology of cells on titanium disks (Fig. 3).

Effects of specific-COX-2 inhibitor NSAIDs on proliferation and differentiation of osteoblasts are inconsistent. The present study agreed with Evan and Butcher [10] that non-specific and specific COX-2 inhibitor NSAIDs inhibited growth of osteoblasts but disagreed with Boyan and co-workers [11] that specific COX-2 inhibitor did not have any effects on proliferation of osteoblasts on smooth titanium surface. Evan and Butcher[10] reported that indomethacin 0.1-1.0 μM inhibited proliferation of osteoblasts on cell culture plate. Boyan and co-workers[11] found that specific COX-2 inhibitor, NS-398, 1 and 10 μM, did not have any effects on proliferation of osteoblasts on smooth surface titanium disks.

Regarding the effects on differentiation of cells, the current findings agreed previous reports[11] that specific Cox-2 inhibittor NSAIDs, celecoxib, does not interfere with progress of osteoblastic differentiation into mature stage (Fig 10). It was found that indomethacin and celecoxib did not clearly show their effects on ALP and osteocalcin levels (Figs 9 and 10). A tendency to promote ALP activity of celecoxib in dose and exposure time dependent manners was found in plateau phase (Fig 9).

A variation of effects of NSAIDs on growth and differentiation of osteoblasts in different drug treatment time and stages of cell growth suggested that effects of both non-specific and specific cox-2 inhibitor NSAIDs on cell growth was influenced by drug treatment time and stages of cell growth (Fig 4-6). The present study

demonstrated influence of stages of cell growth on effects of celecoxib on growth and differentiation of osteoblasts by adding the medications in culture medium at different stages of cell growth, *static*, *log* and *plateau* phases which are comparable to three stages of osteoblastic differentiation including proliferation and matrix formation and mineralization[38]. Thus an experimental condition in the current study was different from most of the previous studies, where osteoblasts were treated with the medications during days 1 - 4 after cell seeding[10,11,39,40], which was comparable to a *static* phase in the present study. Only a study of Ho et al., 1999[40] reported effects of conventional NSAIDs, indomethacin, on osteoblasts in different stages of cell growth on culture-days 2, 10, 15 and 20, which is supported by the present study that effects of treated drugs in each experimental phase were different and indomethcin decreased cell growth (Figs 4 and 6). Furthermore, the inhibitory effect of indomethacin was greater than celecoxib in a stage of rapid cell growth or *log* phase (Fig 5).). In addition, a discrepancy of the results from previous studies may be results of inconsistent experimental conditions such as a variation in drug treatment period, condition of culture medium and stages of cell growth. It was an advantage of the current study that the investigation was performed in serum free culture condition in which this condition was not only avoiding interference of growth factors but also providing a low protein binding condition for the drug, thus, potency of the drug might not be obscured.

Dose dependent effects of conventional and specific COX-2 inhibitors NSAIDs are clearly demonstrated in most studies[10,40]. Inhibitory effect of indomethacin and celecoxib in dose and exposure time dependent manners found in the present study supported previous *in vivo* studies reporting inhibitory effects of specific COX-2 inhibitors, celecoxib and rofecoxib on bone healing[41] and bone

ingrowth into porous chrome-cobalt[42] and titanium implants[7,43] by inhibiting proliferation of cells in dose and exposure time dependent manner.

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 50-200 mg or 284 ng/ml in plasma[44] and celecoxib is 100-200 mg/day for osteoarthritis, 200-400 mg/day for rheumatoid arthritis and 200 mg/day for acute pain. With a half life of 12 hours, plasma levels of therapeutic doses of 100, 400, 800 mg/day are equivalent to 1.7, 3.1 and 9.2 μM, respectively[45]. Therefore an undertaking of celecoxib in therapeutic doses of 100 – 400 mg/day could pose adverse effects on growth of osteoblasts on 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 were tended to be in a lower level than indomethacin (Figs 4 and 5).

As it is known that conventional NSAIDs, indomethacin, inhibit functions of both COX-1 and COX-2 (IC50 ratio COX-2/COX-1 = 0.01) and affinity of specific COX-2 inhibitor, celecoxib, to COX-2 (IC50) is much higher than to COX-1 (IC50 ratio COX-2/COX-1 = 375)[46], significant decrease of PGE_2 levels found in the present study (Fig 11) implied that treated indomethacin and celecoxib were able to inhibit the function of cycloxygenase pathway by blocking synthesis of the cyclooxygenase enzymes and the effects were influenced by stages of cell growth and doses of celecoxib (Fig 11). The finding agreed with Coetzee and coworkers[47] reporting that specific COX-2 inhibitors, NS-398 0.1 μM and indomethacin 1.0 μmolar inhibited PGE_2 production of MG63 and MC3T3-E1.

However, changing of cell growth, ALP activity and osteocalcin levels did not correspond with decreasing of PGE_2 synthesis found in all experimental phases.

Thus, it was unlikely that effects of NSAIDs on growth and differentiation of osteoblasts were results of reduction of PGE_2 function. This assumption is suppoted by Cheng and co-workers[48] that the effect of celecoxib on 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[49]. The results led to a postulation that effects of specific COX-2 inhibitor on growth and differentiation of osteoblasts did not critically depend on a reduction of PGE_2 synthesis.

It is well known that osteointegration involves a complex series of events and is tied to discrete phases of osteogenesis including osteogenic differentiation, matrix production, mineralization and remodeling[50], thus growth and differentiation of osteoblasts are one of prime factors determining success or failure of osteointegration. In addition, osteoblatic differentiation is temporally regulated and is closely related to stages of cell growth[51]. 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 inhibitor could interfere with osteointeingration of dental implant and the effects of NSAIDs on different stages of osteointegration could be different. Effects of specific COX-2 inhibitor, celecoxib, are expected to be lower than indomethacin or conventional NSAIDs.

CONCLUSIONS

The results of this study demonstrated that indomethacin and celecoxib in therapeutic doses were able to inhibit growth of osteoblasts on smooth titanium surface in dose and time dependent manner and the effects were influenced by stages

of cell growth. The findings suggested that undertaking of celecoxib in therapeutic doses of 200-400 mg/day of more than 3 days could jeopardize osteointegration by inhibiting proliferation of cells in proliferative stage of osteointegration, particular in high doses and longer exposure time.

REFERENCES

1. Devlin T. Lipid metabolism II:pathwaysof metabolism of special lipid. In: Thomas M.Devlin. (eds). textbook of biochemistry with clinical corrrelations. philadelphia, pennsylvania: wiley-liss, 2006:432-432

2. Einhorn TA. Do inhibitors of cyclooxygenase-2 impair bone healing? J Bone Miner Res 2002;17:977-978.

3. Crofford LJ. COX-1 and COX-2 tissue expression: implications and predictions. J Rheumatol Suppl 1997;49:15-19.

4. Gajraj NM. The effect of cyclooxygenase-2 inhibitors on bone healing. Reg Anesth Pain Med 2003;28:456-465.

5. Dumont AS, Verma S, Dumont RJ,Hurlbert RJ. Nonsteroidal anti-inflammatory drugs and bone metabolism in spinal fusion surgery: a pharmacological quandary. J Pharmacol Toxicol Methods 2000;43:31-39.

6. Nagata T, Kido J, Hamasaki A, Ishida H,Wakano Y. Regulation of glycosaminoglycan synthesis by parathyroid hormone and prostaglandin E2 in cultured dental pulp cells. J Endod 1991;17:594-597.

7. Goodman S, Ma T, Trindade M, Ikenoue T, Matsuura I, Wong N, Fox N, Genovese M, Regula D,Smith RL. COX-2 selective NSAID decreases bone ingrowth in vivo. J Orthop Res 2002;20:1164-1169.

8. Simmons DL, Botting RM,Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev 2004;56:387-437.

9. Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN,O'Keefe RJ. Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. J Clin Invest 2002;109:1405-1415.

10. Evans CE,Butcher C. The influence on human osteoblasts in vitro of non-steroidal anti-inflammatory drugs which act on different cyclooxygenase enzymes. J Bone Joint Surg Br 2004;86:444-449.

11. Boyan BD, Lohmann CH, Sisk M, Liu Y, Sylvia VL, Cochran DL, Dean DD,Schwartz Z. Both cyclooxygenase-1 and cyclooxygenase-2 mediate osteoblast response to titanium surface roughness. J Biomed Mater Res 2001;55:350-359.

12. El Desoky,Ehab S. Pharmacotherapy of rheumatoid arthritis: an overview. Current Therapeutic Research 2001;62:92-112.

13. Rehman Q,Sack KE. When to try COX-2-specific inhibitors. Safer than standard NSAIDs in some situations. Postgrad Med 1999;106:95-2, 105.

14. Cutts C, LaCaze A,Tett S. A clinical audit of the prescribing of celecoxib and rofecoxib in Australian rural general practice. Br J Clin Pharmacol 2002;54:522-527.

15. Sarzi-Puttini P, Cimmino MA, Scarpa R, Caporali R, Parazzini F, Zaninelli A, Atzeni F,Canesi B. Osteoarthritis: an overview of the disease and its treatment strategies. Semin Arthritis Rheum 2005;35:1-10.

16. Tenenbaum H. Biology perspectives related to osseointegration. In: Zarb G., Lekholm U., Albrektsson T. and Tenenbaum H. (eds). Aging, osteoporosis, and dental implants. 1996:

17. Rosa AL,Beloti MM. Rat bone marrow cell response to titanium and titanium alloy with different surface roughness. Clin Oral Implants Res 2003;14:43-48.

18. Bachle M,Kohal RJ. A systematic review of the influence of different titanium surfaces on proliferation, differentiation and protein synthesis of osteoblast-like MG63 cells. Clin Oral Implants Res 2004;15:683-692.

19. Lumbikanonda N,Sammons R. Bone cell attachment to dental implants of different surface characteristics. Int J Oral Maxillofac Implants 2001;16:627-636.

20. Mustafa K, Wroblewski J, Hultenby K, Lopez BS,Arvidson K. Effects of titanium surfaces blasted with TiO2 particles on the initial attachment of cells derived from human mandibular bone. A scanning electron microscopic and histomorphometric analysis. Clin Oral Implants Res 2000;11:116-128.

21. Mustafa K, Wennerberg A, Wroblewski J, Hultenby K, Lopez BS,Arvidson K. Determining optimal surface roughness of TiO(2) blasted titanium implant material for attachment, proliferation and differentiation of cells derived from human mandibular alveolar bone. Clin Oral Implants Res 2001;12:515-525.

22. Okazaki Y, Rao S, Tateishi T,Ito Y. Cytocompatibility of various metal and development of new titanium alloys for medical implants. Materials Science and Engineering A243 1998;A243 :250-256.

23. St-Pierre JP, Gauthier M, Lefebvre, LP,Tabriziana M. Three-dimensional growth of differentiating MC3T3-E1pre-osteoblasts on porous titanium scaffolds. Biomaterials 2005;26 :7319-7328.

24. Tanaka SM, Lib J, Duncan RL, Yokota H, Burr DB,Turner CH. Effects of broad frequency vibration on cultured osteoblasts. Journal of Biomechanics 2003;36:73-80.

25. Quarles LD, Yohay DA, Lever LW, Caton R,Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res 1992;7:683-692.

26. Sudo H, Kodama HA, Amagai Y, Yamamoto S,Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 1983;96:191-198.

27. Lohmann CH, Sagun R, Jr., Sylvia VL, Cochran DL, Dean DD, Boyan BD,Schwartz Z. Surface roughness modulates the response of MG63 osteoblast-like cells to 1,25-(OH)(2)D(3) through regulation of phospholipase A(2) activity and activation of protein kinase A. J Biomed Mater Res 1999;47:139-151.

28. Franceschi RT,Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res 1992;7:235-246.

29. Mustafa K, Rubinstein J, Lopez BS,Arvidson K. Production of transforming growth factor beta1 and prostaglandin E2 by osteoblast-like cells cultured on titanium surfaces blasted with TiO2 particles. Clin Oral Implants Res 2003;14:50-56.

30. Stephansson SN, Byers BA,Garcia AJ. Enhanced expression of the osteoblastic phenotype on substrates that modulate fibronectin conformation and integrin receptor binding. Biomaterials 2002;23:2527-2534.

31. Attawia MA, Herbert KM, Uhrich KE, Langer R,Laurencin CT. Proliferation, morphology, and protein expression by osteoblasts cultured on poly(anhydride-co-imides). J Biomed Mater Res 1999;48:322-327.

32. Ku CH, Pioletti DP, Browne M,Gregson PJ. Effect of different Ti-6Al-4V surface treatments on osteoblasts behaviour. Biomaterials 2002;23:1447-1454.

33. Rea SM, Brooks RA, Schneider A, Best SM,Bonfield W. Osteoblast-like cell response to bioactive composites-surface-topography and composition effects. J Biomed Mater Res B Appl Biomater 2004;70:250-261.

34. Lee TM, Chang E,Yang CY. Attachment and proliferation of neonatal rat calvarial osteoblasts on Ti6Al4V: effect of surface chemistries of the alloy. Biomaterials 2004;25:23-32.

35. Ramires PA, Romito A, Cosentino F,Milella E. The influence of titania/hydroxyapatite composite coatings on in vitro osteoblasts behaviour. Biomaterials 2001;22:1467-1474.

36. Zhu X., Chen J., ScheidelerL.Reichl R,Gerstorfer J.G. Effects of topography and composition of titanium surface oxides on osteoblast responses. Biomaterials 2004;25: 4087-4103.

37. Hutchings S. basic methods. In: Jakoby, Willium B. and Date. (eds). Cell Culture. San Diego,California: Academic press, Inc., 1979:94-109

38. Aubin JE. Advances in the osteoblast lineage. Biochem Cell Biol 1998;76:899-910.

39. Chang JK, Chuang LY, Ho ML,Wang GJ. Effects of nonsteroidal anti-inflammatory drugs on transforming growth factor-beta expression and bioactivity in rat osteoblast-enriched culture. Kaohsiung J Med Sci 2003;19:278-288.

40. Ho ML, Chang JK, Chuang LY, Hsu HK,Wang GJ. Effects of nonsteroidal anti-inflammatory drugs and prostaglandins on osteoblastic functions. Biochem Pharmacol 1999;58:983-990.

41. Simon AM, Manigrasso MB,O'Connor JP. Cyclo-oxygenase 2 function is essential for bone fracture healing. J Bone Miner Res 2002;17:963-976.

42. Trancik T, Mills W,Vinson N. The effect of indomethacin, aspirin, and ibuprofen on bone ingrowth into a porous-coated implant. Clin Orthop Relat Res 1989113-121.

43. Cook SD, Barrack RL, Dalton JE, Thomas KA,Brown TD. Effects of indomethacin on biologic fixation of porous-coated titanium implants. J Arthroplasty 1995;10:351-358.

44. Keller J, Bunger C, Andreassen TT, Bak B,Lucht U. Bone repair inhibited by indomethacin. Effects on bone metabolism and strength of rabbit osteotomies. Acta Orthop Scand 1987;58:379-383.

45. McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA,FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci U S A 1999;96:272-277.

46. Chai-amnoui P and Akarasereenont P. New trend in NSAIDs therapy: Cox-2 inhibitors and clinical NSAIDs. ruen-kaew kanpim, 2000.

47. Coetzee M, Haag M, Claassen N,Kruger MC. Stimulation of prostaglandin E2 (PGE2) production by arachidonic acid, oestrogen and parathyroid hormone in MG-63 and MC3T3-E1 osteoblast-like cells. Prostaglandins Leukot Essent Fatty Acids 2005;73:423-430.

48. Cheng AS, Chan HL, Leung WK, Wong N, Johnson PJ,Sung JJ. Specific COX-2 inhibitor, NS-398, suppresses cellular proliferation and induces apoptosis in human hepatocellular carcinoma cells. Int J Oncol 2003;23:113-119.

49. Damrongsri D, Geva S, Salvi GE, Williams RC, Limwongse V,Offenbacher S. Cyclooxygenase-2 inhibition selectively attenuates bone morphogenetic protein-6 synthesis and bone formation during guided tissue regeneration in a rat model. Clin Oral Implants Res 2006;17:38-47.

50. Balshi TJ. Implant rehabilitation of a patient after partial mandibulectomy: a case report. Quintessence Int 1995;26:459-463.

51. Choi JY, Lee BH, Song KB, Park RW, Kim IS, Sohn KY, Jo JS,Ryoo HM. Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells. J Cell Biochem 1996;61:609-618.

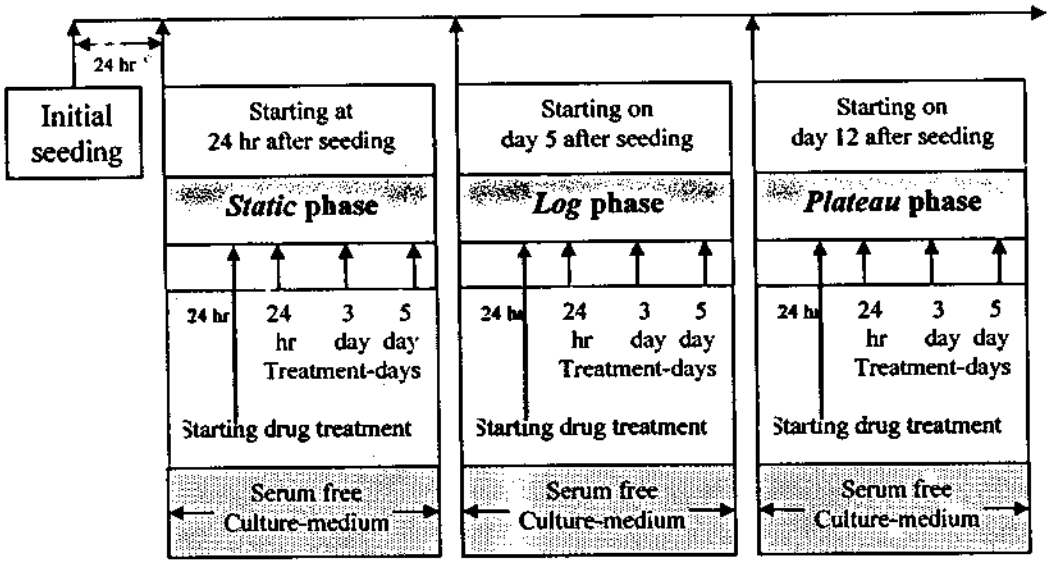

Fig 1 An overview of experimental phases and drug treatment scheme

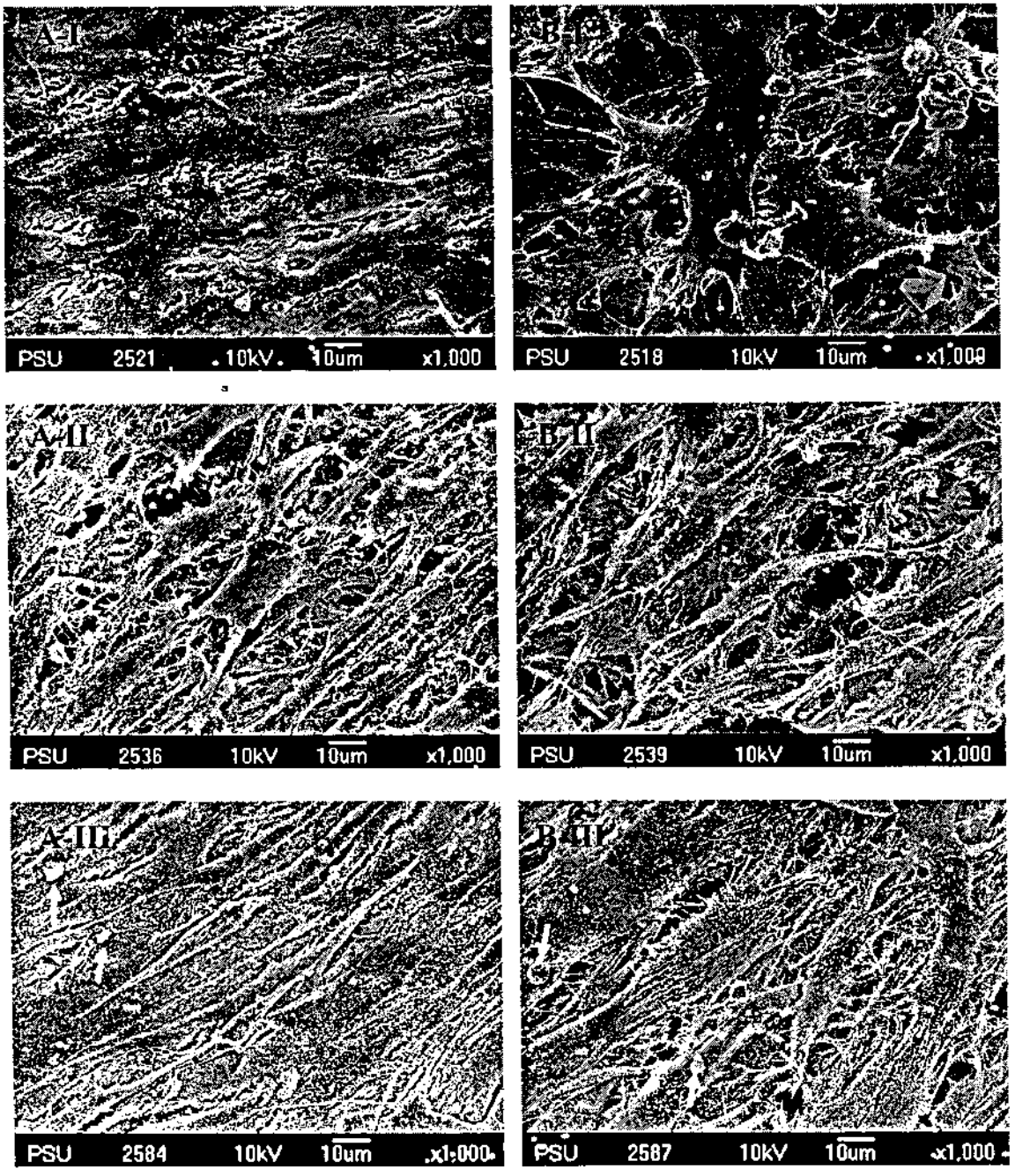

Fig 2 SEM images of MC3T3-E1 on the surface of titanium disk cultured for 7 days (on drug treatment day 5) in culture medium with (A) and without serum (B). (I) *Static*, (II) *Log*, and (III) *Plateau* phases. ↑ extracellular matrix (Original magnification x1000).

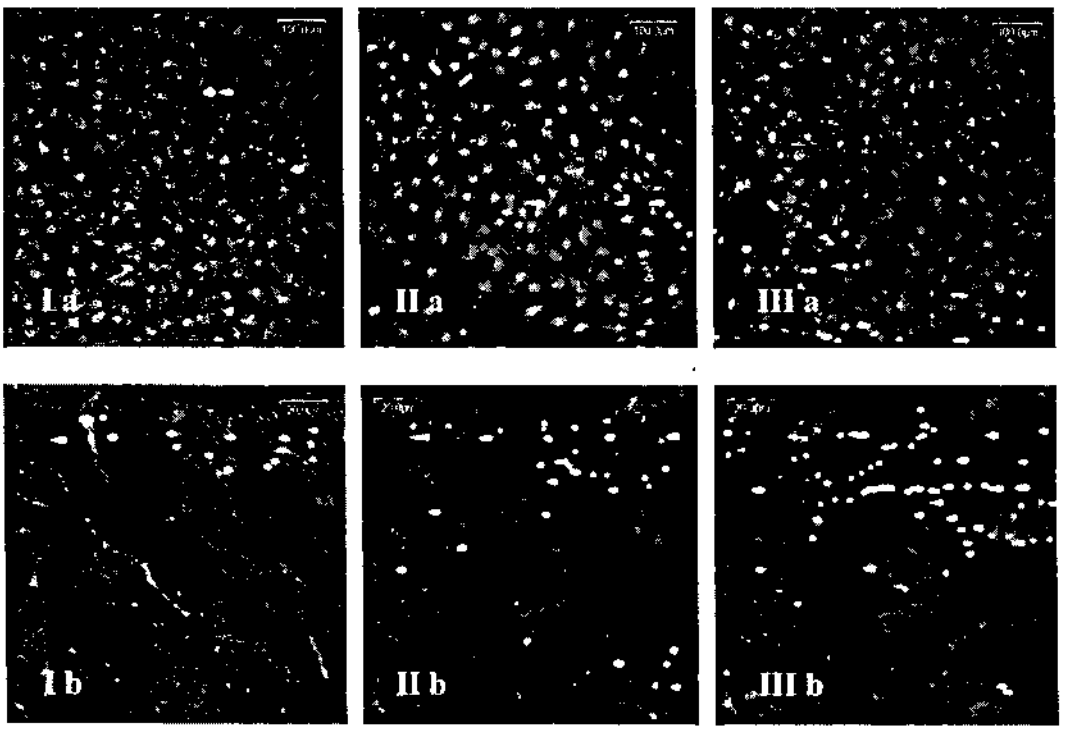

Fig 3 CLSM images of MC3T3-E1 on surface of titanium disks cultured for 7 days (on drug treatment day 5). (I) Group E: a control group without drug treatment; (II) Group A: 0.1 μM indomethacin, and (III) Group C: 3.0 μM celecoxib {(a) original magnification x 200, and (b) original magnification x1000}

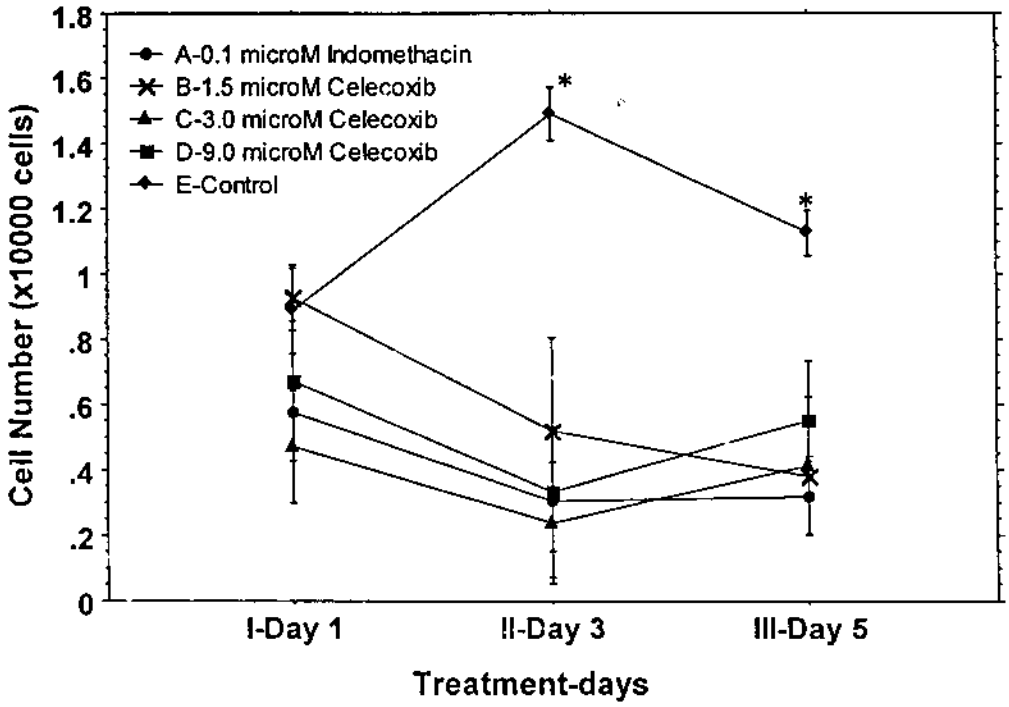

Fig 4 Growth of cells in *Static* phase on treatment days 1, 3 and 5. $^{*}P<0.05$; significant against all experimental groups, Groups A-D (mean ± SE, n = 6)

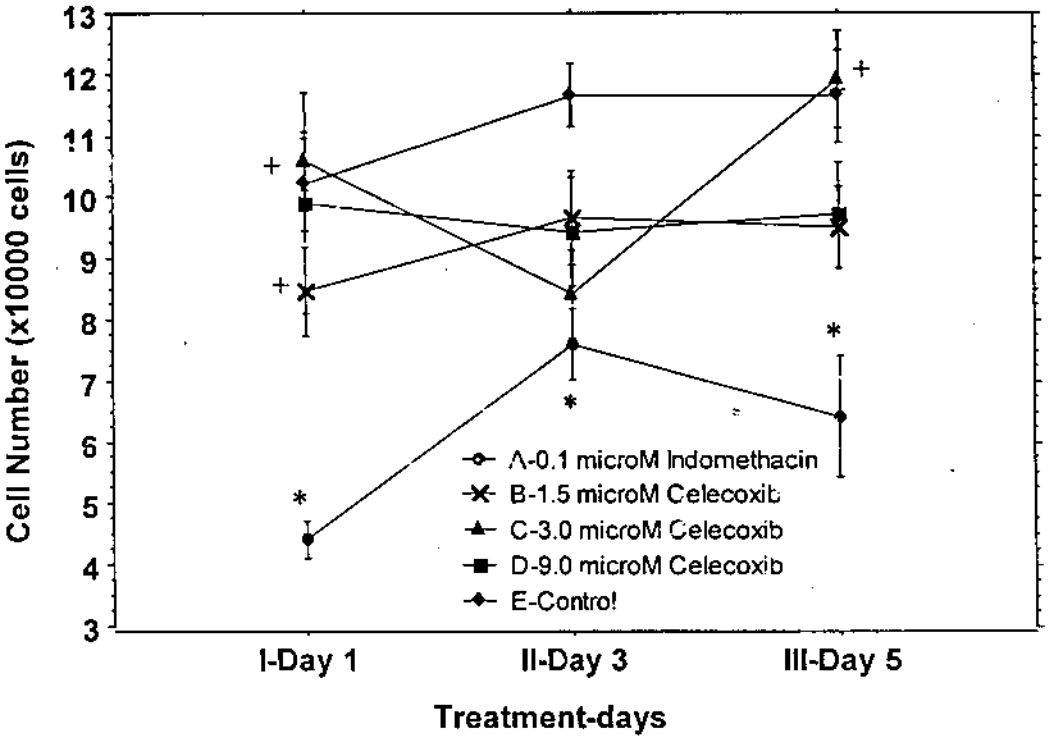

Fig 5 Growth of cells in *Log* phase on treatment days 1, 3 and 5. $^{*}P<0.05$; significant against control, Group E. $^{+}P<0.05$; significant against Group A: 0.1 μM indomethacin (mean ± SE, n = 6).

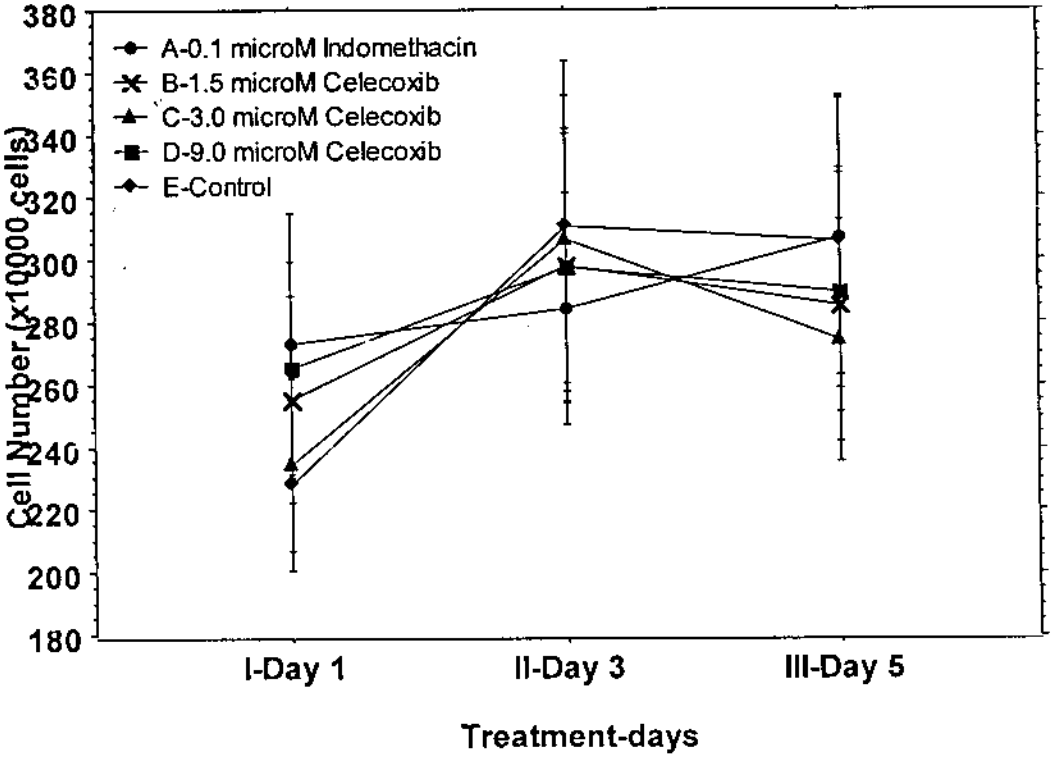

Fig 6 Growth of cells during medication treatment in *Plateau* phase Numbers of cells among groups were not significantly different (mean ± SE, n = 6).

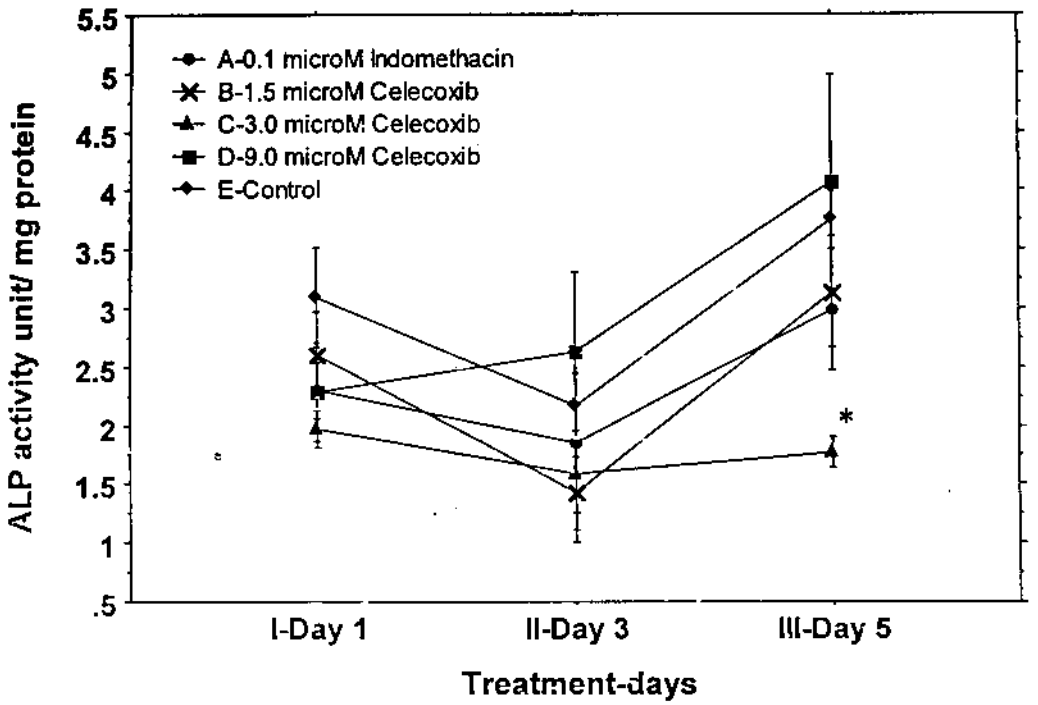

Fig 7 Alkaline phosphatase (ALP) activity of cells on titanium disks in *Static* phase on treatment days 1, 3 and 5. $^{*}P<0.05$; significant against control, Group E (mean ± SE, n = 6).

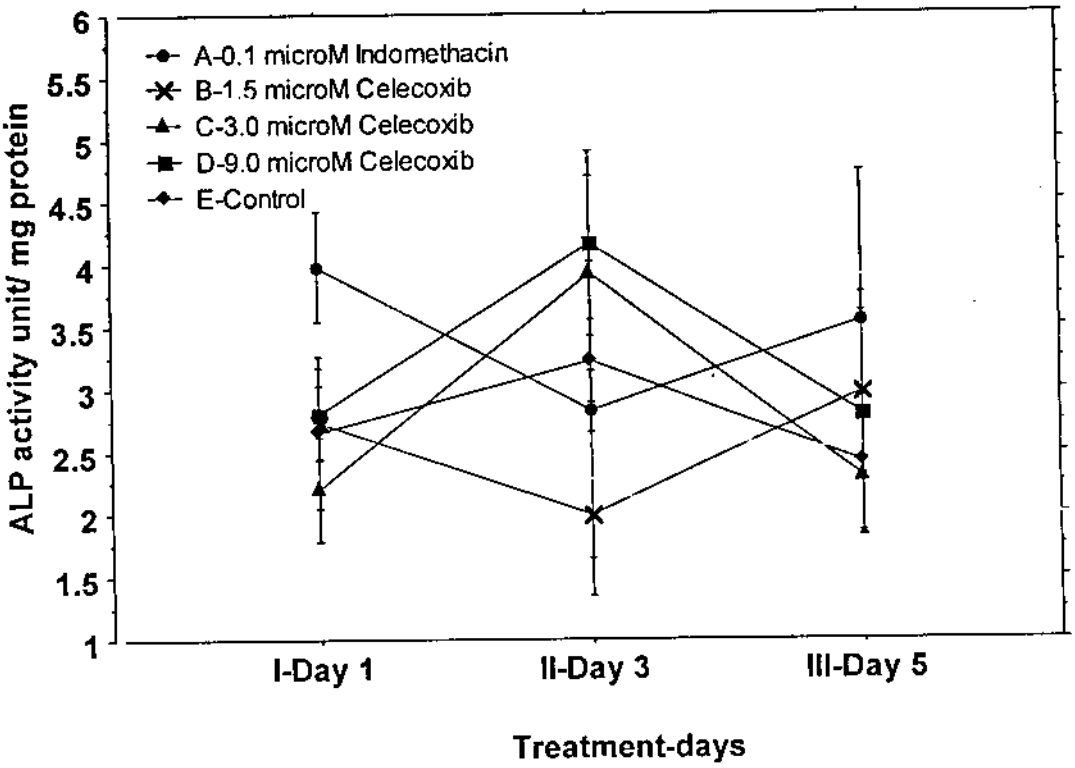

Fig 8 Alkaline phosphatase (ALP) activity of cells on titanium disks in *Log* phase on treatment days 1, 3 and 5. The activity of cells among groups were not significantly different (mean ± SE, n = 6)

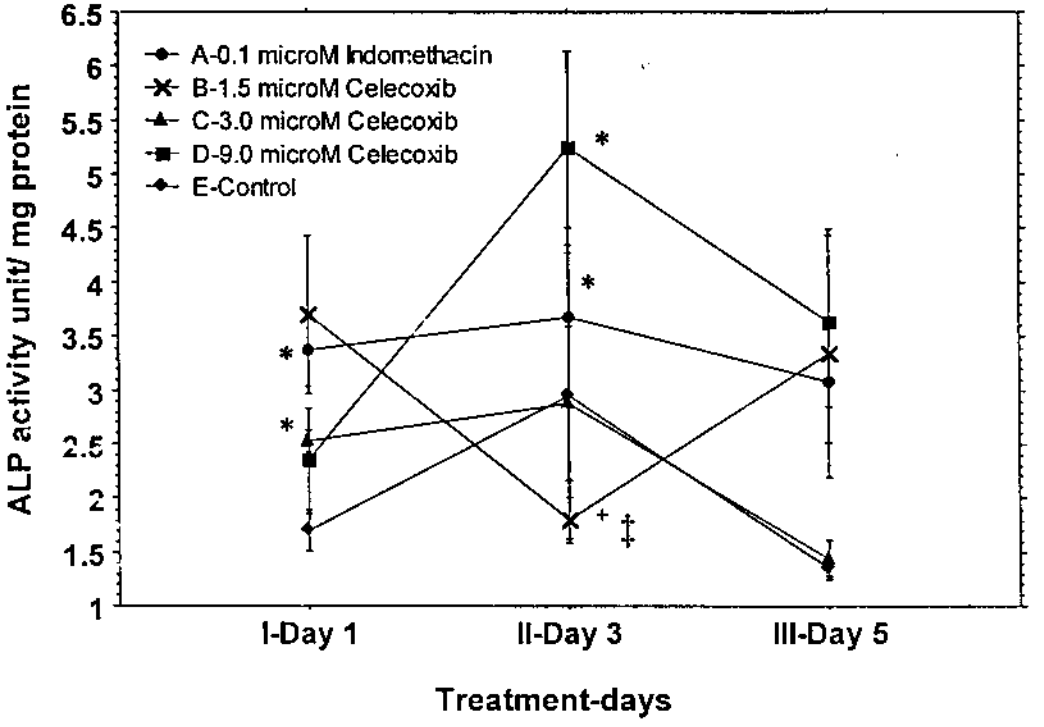

Fig 9 Alkaline phosphatase (ALP) activity of MC3T3-E1 on titanium disks during drug treatment in *Plateau* phase. $^{*}P<0.05$; significant against control, Groups E. $^{+}P<0.05$: significant against Group A: 0.1 μM indomethacin. $^{‡}P<0.05$; significant against Group D: 9.0 μM celecoxib (mean ± SE, n = 6).

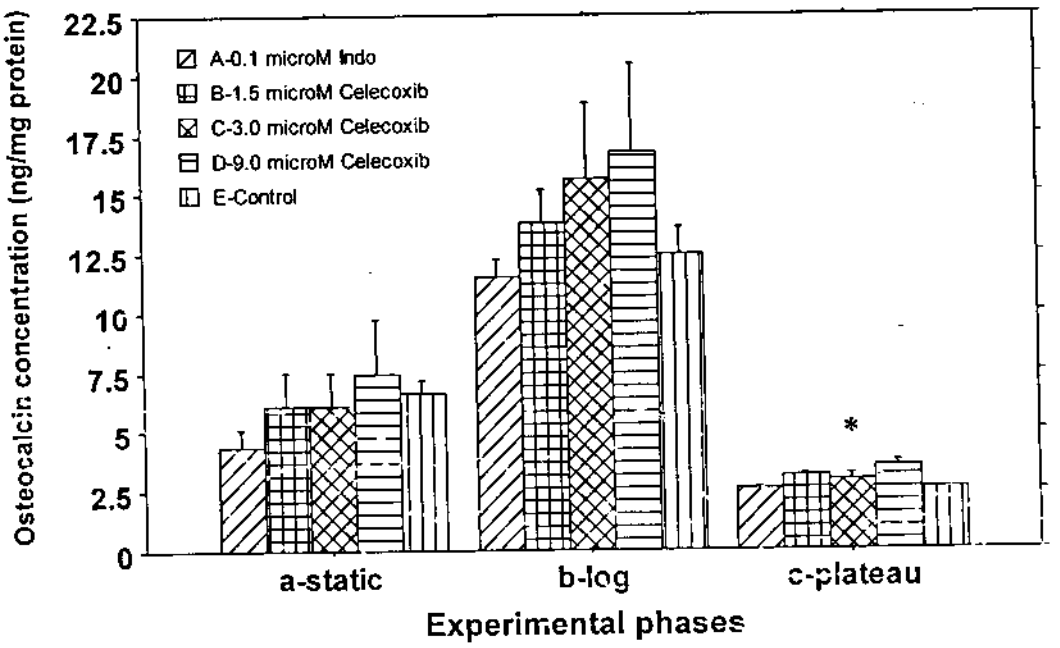

Fig 10 Levels of osteocalcin in culture medium on treatment-day 5 in experimental *static, log and plateau* phases (mean ±SE, n=3) $^{*}P<0.05$; all groups in *plateau* phase significant against all groups *in static* and *log* phases (mean ± SE, n = 3).

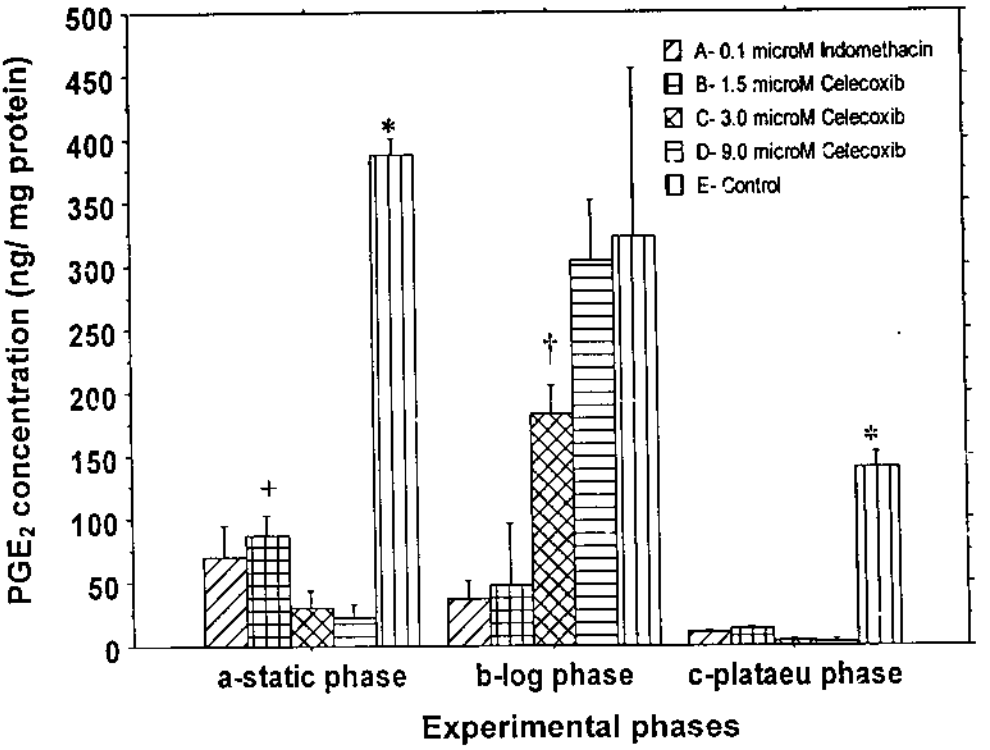

Fig 11 Levels of PGE_2 in culture medium on treatment-day 5 in each experimental phase, *static*, *log* and *plateau* phases; $^{*}P<0.05$ significant against all experimental groups, Groups A-D. $^{+}P<0.05$ significant against Group B in plateau phase. $^{\dagger}P<0.05$ significant against Group C in *static* phase. (mean $\pm$SE, n=3).