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Effect of hydroxyapatite particle size on myoblasts and fibroblasts

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After surgery, the bone and soft tissues around integrated biomaterials can be adversely affected by implant-related factors acting over a period of years. However, few studies have directly addressed the effects upon the adjacent soft tissue. The present study was designed to test the biological effects of various sized hydroxyapatite (HA) particles on myoblasts and fibroblasts. Both the myoblasts and fibroblasts were mixed in *in vitro* culture with 0.1% (1 mg ml^{-1}) of various sized HA particles (0.5–3.0, 37–63, 177–250, 420–841 μm) for 1 h, 3 h, 1 day, 3 days and 7 days to test their effects on the cell culture. The results show that adding HA particles into a cell culture can decrease the cell count significantly. The transforming growth factor-β1 (TGF-β1) concentrations in the culture medium decreased significantly on addition of HA particles. When calculated as a ratio to the cell number, the TGF-β1 titre increased most significantly in the groups of medium-sized particles. The prostaglandin E_2 (PGE_2) concentrations in the medium increased significantly. The changes in TGF-β1 and PGE_2 concentrations with the smallest particles were most significant and persisted longer. The inhibitory effects of the HA particles on the cell culture were mediated by the increased synthesis of PGE_2. Caution should be exercised before considering the use of an HA product which could easily break down into a fine powder.

Keywords: Hydroxyapatite, particle size, myoblasts, fibroblasts

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Recent advances in orthopaedic surgery can be attributed to the revolution in biomaterials. During the last decade, a large number of biomaterials have been proposed as artificial bone fillers for repairing bone defects. The materials most widely used in clinical medicine have been hydroxyapatite (HA) ceramics[1,2]. The inertness of biomaterials is relative: even materials considered inert in bulk form are capable of eliciting an inflammatory reaction in particulate form[3]. It has been reported that the use of porous HA blocks results in an unacceptably high failure rate in clinical applications[4].

After surgery, osteo-integration is employed. However, the bone and soft tissues around the integrated biomaterials can be adversely affected by implant-related factors acting over a period of years[5]. The effects of implants on bony tissue have been investigated[6,7]. However, few studies directly addressed the effects upon the adjacent soft tissue. By testing the cells that give rise to particular tissues rather than the tissue itself, the biological effects of biomaterials on the soft tissue can be elucidated.

Host tissue responses to these materials are generally assessed by morphological and histological examinations at the implant site to evaluate their biocompatibility[8]. It is difficult to examine the *in vivo* reaction of a specific cell to the substrate because numerous cell populations and chemical factors are involved in implantation. In order to determine the sequences of events and the parameters influencing the interactive process, a model of the cell culture in the presence of biomaterials is of great importance.

It has been recognized for a long time that particulate debris around an orthopaedic implant has an adverse effect on the surrounding tissues[9]. Initially, it was assumed that the damaging effect of the particles was essentially similar to that of the bulk material except that the particles have a larger surface area to interact with the surrounding tissues. More recently, it has been suggested that the adverse effects depend more on the particulate nature of the material than its chemical biocompatibility[10]. However, there is not yet any general understanding of the mechanisms by which particulate materials might exert a harmful effect greater than that of the parent material. Cytokines are polypeptide mediators which can be produced by a variety of cells. The cytokine networks can regulate cellular events and have distinct interactions with tissue. Through the production of cytokines the cells send out messages on a local or systemic level[11]. The present study was designed to test the mechanism of biological effects of various sized HA particles on myoblasts and fibroblasts.

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MATERIALS AND METHODS

Material tested

Hydroxyapatite (HA: $Ca_5(PO_4)_6(OH)_6$, Merck, Germany) powder was prepared as follows. The HA powders were placed in a platinum crucible and heated up to 1250°C at a heating rate of 3°C min^{-1} in a conventional Ni–Cr coiled furnace and then maintained in air for 1 h after the sintering temperature of 1250°C was reached. The sintered particles were ground and separated with mesh into various grain sizes of about 0.5–3.0, 37–63, 177–250 and 420–841 μm. HA particles were spherical in shape at various grain sizes according to scanning electron microscopic observation. Trace elements that might be connected with biocompatibility were detected by atomic absorption analysis. The concentrations of the trace elements were much lower than the maximum tolerable level. Prior to use, they were processed by dry heat sterilization (130°C, 40 min).

Enzyme digestion of myoblasts and fibroblasts

The method of enzymatic digestion of myogenic cells was similar to that of Bischoff,[12] with some modification. Briefly, newborn Wistar rats of both sexes were anaesthetized with pentothal (25 mg per 100 g, intraperitoneal injection). The rat was prepared and disinfected. The skeletal muscles of the hind limbs were excised and rinsed several times with sterile normal saline solution. Muscle tissue was then minced into 1-mm fragments. The tissue fragments were trypsinized twice at 37°C for 30 min with 0.25% trypsin (Gibco, UK). After each digestion, the fragments were removed by low speed centrifugation (500 **g** for 1 min) and transferred to fresh medium for further dissociation. The supernatant containing the liberated cells was centrifuged again (1500 **g** for 3 min) to pellet the cells and large debris while leaving most of the myofibrils in suspension. The pellet was suspended in fresh medium and aliquots were transferred to culture dishes. Enrichment of the myoblast population was accomplished by pre-plating the cells at 37°C for 30 min on a non-coated dish. The cells pre-plated at the bottom of the dish are mainly fibroblasts. This study received prior approval of the National Taiwan University Medical College's Animal Research Committee.

Experimental procedures

In the experiment, confluent rat myoblast or fibroblast cultures were seeded into six $3.0 \times 3.0\,mm^2$ tissue culture wells (seeding density of 1×10^5 cells per well). The culture medium used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco, UK), 100 units ml^{-1} penicillin G sodium and 100 mg ml^{-1} streptomycin (Gibco, UK). The dishes were incubated at 37°C in an atmosphere supplemented with 5% CO_2 for 24 h to facilitate the attachment of myoblasts and fibroblasts. The medium was then removed and washed twice with phosphate-buffered saline (PBS) solution. DMEM supplemented with ITS (insulin, 10 μg ml^{-1}, transferrin, 5 μg ml^{-1} and sodium selenite, 5×10^{-3} μg ml^{-1}; Sigma Corp.) was mixed with 0.1% (1 mg ml^{-1}) of various sized HA particles. The day of plating was considered as the zero day of culture. The test media were removed from the wells after 1 h, 3 h and 1, 3 and 7 days, divided into sections of 500 μl with Eppendorf, and deep frozen at −80°C till further analysis.

Cell count

After removal of the medium, the wells were fixed with 3% formaldehyde in 0.1 M PBS (pH 7.4). The samples were stained with haematoxylin–eosin and then observed by inverted microscopy (Olympus, IMT-II, Japan). The cellularity of the control and experimental wells was determined by an MICD image analysing system (MICD Software Series, Image Research Inc., Ontario, Canada). Briefly, the mean cell population in four randomly selected high-power fields (0.06 mm^2) was measured by counting the number of nuclei caught by CCD-72 camera (Dage-Mill Inc., Michigan, USA) through the microscope. Then the cell count per well was calculated by multiplying by the area of each well (940 mm^2 per well).

Surface area

For the measurement of the surface area of the cells, more than 100 cells were counted in randomly selected high-power fields for each well. After calibration with a standard, the mean surface area of the cells in each well can be automatically calculated by using the MICD image analysing system.

Transforming growth factor-β1 (TGF-β1) in culture medium

The production of TGF-β1 in the culture medium was analysed by enzyme-linked immunosorbent assay (ELISA) methods. Briefly, 200 μl of standard or sample were added per well. The tested samples were incubated for 3 h at room temperature on the benchtop. Then 200 μl of TGF-β1 (R & D System, Inc., USA) conjugate were added. The mixture was incubated for 1.5 h at room temperature. Substrate solution (200 μl) was then added to each well. The reaction was stopped and read with a Microelisa reader (Emax Science Corp., USA) at 450 nm after 20 min incubation.

Prostaglandin E_2 (PGE_2) in culture medium

The production of prostaglandin E_2 (PGE_2) in the culture medium was also analysed by ELISA methods. Briefly, 50 μl of standard PGE_2 (Cayman Chemical Company, MI, USA) or sample were added per well. The tested samples were incubated for 18 h at room temperature on the benchtop. Then 200 μl of Ellman's reagent were added to each well and the mixtures incubated for 1.5 h at room temperature. The reaction was stopped and read with a Microelisa reader at 405 nm.

Statistical analysis

The effects of various sized HA particles were evaluated by an analysis of variances (ANOVA) statistical method. The *post hoc* test performed was Bonferroni's test. The level of statistical significance is defined as $P < 0.05$.

RESULTS

Cell count

Adding HA particles into a cell culture can significantly affect the cell count. The cell populations of the control and various sized HA particles are summarized in *Table 1*. The changes in cell population of various preparations at 1 h, 3 h, 1 day, 3 days and 7 days were all statistically significant ($P < 0.0001$ by the ANOVA test) (*Table 1*).

The cell population of the control increased persistently except at 3 h after passage of cells. When cultured with HA particles, the cell populations of all experimental groups were significantly lower than that of the control ($P < 0.05$) (*Table 1*). The changes in cell population were quite similar between the groups with HA of 37–63, 177–250 and 420–841 μm and lower than that of control. The cell population of the smallest sized HA (0.5–3.0 μm) was even lower than that of the control group and groups with other sized HA particles (*Table 1*). The cell population of the smallest sized HA has the fewest cell populations (*Figure 1*).

Surface area

The mean surface area of cells in various preparations is shown in *Table 2*. The mean surface area of myoblasts in the control group increased and then decreased on the first day and then increased gradually up to the 7th day of culture (*Table 2*). The mean surface area of myoblasts in the groups cultured with larger sized HA particle (420–841 μm) increased on the first day of culture, decreased till the 3rd day of culture, and then increased again till the 7th day of culture (*Table 2*). The mean surface area of myoblasts in the smaller size (177–250, 37–63 and 0.5–3.0 μm) increased in the first 3 days, then decreased gradually till the 7th days' culture (*Table 2*). The differences in the mean surface areas of the myoblasts were always statistically significant between the control group and the various tested experimental groups.

The mean surface area of the fibroblasts in the control group decreased and then increased in the initial 3 days of culture, then decreased gradually till the 7th day of culture (*Table 2*). The mean surface area of fibroblasts in the groups cultured with various sized HA particles (420–841, 177–250 and 36–63 μm) increased on the first day of culture, and then increased again till the 7th day of culture (*Table 2*). The differences in mean surface area of the fibroblasts were always statistically significant between the control group and various tested experimental groups.

Transforming growth factor-β1 (TGF-β1) in culture medium

The transforming growth factor-β1 (TGF-β1) concentrations in the culture medium were significantly affected by the addition of HA particles, especially when the particles are smaller in size. The TGF-β1 concentrations in culture medium of various preparations are shown in *Table 3*. The changes of TGF-β1 concentration in medium at 1 h and 3 h were low and not statistically significant ($P > 0.05$). The changes of TGF-β1 concentrations in the medium on 1 day, 3 days and 7 days were statistically significant ($P < 0.05$ by ANOVA test) (*Table 3*). The TGF-β1 concentration in culture medium of the smallest sized hydroxyapatite (0.5–3.0 μm) was always significantly lower than that of the control medium 1 day after implantation (*Table 3*). The decreases of TGF-β1 concentration in the culture medium was most significant in the groups with the smallest particles.

When calculated as a ratio to the cell number, the TGF-β1 titre of all particles sizes increased as the culture time increased. The increase in the TGF-β1 titre were most significant in the groups of medium size (i.e. 37–63 and 177–250 μm) particles (*Figure 2*).

Prostaglandin E_2 (PGE_2) in culture medium

The concentrations of prostaglandin E_2 (PGE_2) in culture medium of various preparations are shown in *Table 4*. The changes of PGE_2 concentration were statistically significant ($P < 0.0001$ by ANOVA test). The concentrations of PGE_2 in the experimental medium are always significantly higher than that of control medium (*Table 4*).

Table 1 The changes in cell population of cells cultured with various-sized synthetic hydroxyapatite (HA) particles at a concentration of 1 mg ml^{-1} ($N = 10$)

HA particle size (μm)	Cell count ($\times 10^4$ per well)				
	1 h	3 h	1 day	3 days	7 days
Myoblasts					
A (0.5–3.0)	3.01*** (S.D. 0.94)	0.38*** (S.D. 0.19)	2.91*** (S.D. 0.75)	2.91** (S.D. 4.89)	0.38*** (S.D. 0.28)
B (37–63)	5.45*** (S.D. 1.03)	1.98* (S.D. 0.75)	7.71** (S.D. 1.13)	4.89** (S.D. 4.60)	1.13*** (S.D. 0.47)
C (177–250)	5.55*** (S.D. 0.85)	1.98** (S.D. 0.75)	7.89*** (S.D. 1.32)	5.17* (S.D. 6.54)	0.94*** (S.D. 0.47)
D (420–841)	5.92*** (S.D. 0.56)	1.88** (S.D. 0.85)	8.65*** (S.D. 1.13)	5.17** (S.D. 5.92)	2.26*** (S.D. 1.60)
E Control	7.61 (S.D. 1.13)	2.91 (S.D. 1.32)	12.31 (S.D. 1.79)	10.90 (S.D. 5.63)	35.99 (S.D. 7.24)
P value	2.38×10^{-12}	1.46×10^{-6}	1.81×10^{-14}	0.024	7.19×10^{-29}
Fibroblasts					
A (0.5–3.0)	3.76*** (S.D. 0.56)	0.19*** (S.D. 0.09)	0.47*** (S.D. 0.28)	0.56*** (S.D. 0.66)	1.79*** (S.D. 1.13)
B (37–63)	4.60*** (S.D. 0.66)	0.85*** (S.D. 0.38)	3.20*** (S.D. 0.56)	2.63*** (S.D. 0.94)	3.95*** (S.D. 3.48)
C (177–250)	4.89*** (S.D. 0.47)	1.32* (S.D. 0.94)	3.48*** (S.D. 0.66)	2.91*** (S.D. 1.22)	4.79*** (S.D. 5.36)
D (420–841)	5.45** (S.D. 0.94)	1.22*** (S.D. 0.94)	4.79** (S.D. 0.94)	4.04*** (S.D. 1.79)	12.12*** (S.D. 11.56)
E Control	6.86 (S.D. 0.75)	2.07 (S.D. 0.47)	6.77 (S.D. 1.69)	12.40 (S.D. 1.79)	39.19 (S.D. 11.18)
P value	7.19×10^{-12}	6.77×10^{-9}	2.88×10^{-17}	7.66×10^{-23}	3.13×10^{-14}

All data were analysed by the analysis of variance (ANOVA) test. S.D. = standard deviation.
*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ when compared with control group.

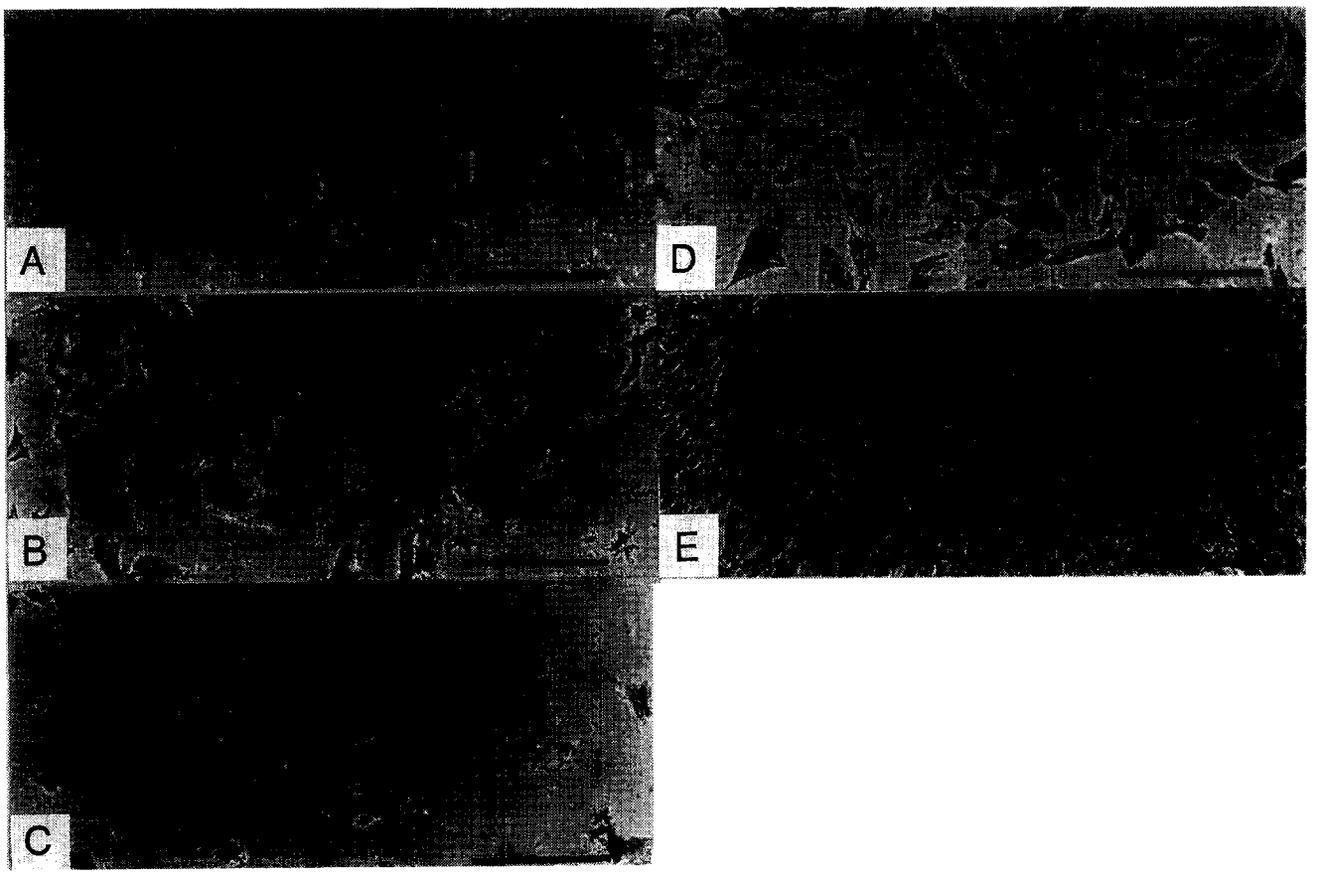

Figure 1 Representative photomicrographs of myoblast cell population after 7 days culture with various-sized hydroxyapatite (HA) bioceramics (A, 0.5–3.0 μm; B, 37–63 μm; C, 177–250 μm; D, 420–841 μm; E, control) (Bars, 100 μm). The addition of HA particles into the myoblast cell cultures can significantly affect the cell count. The changes in cell population were similar between the groups with HA of sizes 37–63, 177–250 and 420–841 μm and lower than that of the control. The cell population of the smallest-sized HA particles (0.5–3.0 μm) is the lowest.

The PGE_2 concentration in the control medium of myoblasts increased in the first 3 days, then decreased gradually till the 7th day. The PGE_2 concentration in the control medium of fibroblasts increased on the first day, decreased gradually till the 3rd day, then increased gradually till the 7th day (*Table 4*). The changes of PGE_2 concentration in culture medium tested with HA particles increased persistently till the 7th day. The

Table 2 The changes in mean surface area of the cells when cultured with various-sized synthetic hydroxyapatite (HA) particles at a concentration of 1 mg ml^{-1} ($N = 10$)

HA particle size (μm)	Surface area (μm^2)				
	1 h	3 h	1 day	3 days	7 days
Myoblasts					
A (0.5–3.0)	1976.8 (S.D. 1181.5)	2031.2 (S.D. 996.9)	1505.3* (S.D. 818.9)	3303.4*** (S.D. 1938.4)	3299.7*** (S.D. 2187.9)
B (37–63)	2500.6*** (S.D. 1076.8)	2617.1* (S.D. 1334.9)	2484.9*** (S.D. 1326.9)	2854.4*** (S.D. 2134.9)	2457.2** (S.D. 1385.3)
C (177–250)	3068.5*** (S.D. 1366.1)	2753.3** (S.D. 1297.2)	2754.4*** (S.D. 1332.6)	2951.4*** (S.D. 2241.5)	2179.3 (S.D. 1528.9)
D (420–841)	2626.9*** (S.D. 1695.3)	2475.8 (S.D. 1172.2)	3125.5*** (S.D. 1688.8)	2319.5* (S.D. 1790.4)	3557.5*** (S.D. 2420.3)
E Control	1815.1 (S.D. 967.1)	2264.6 (S.D. 1072.9)	1786.3 (S.D. 954.3)	1836.6 (S.D. 917.9)	1982.5 (S.D. 1361.1)
P value	3.81×10^{-12}	0.0001	1.72×10^{-7}	1.72×10^{-7}	2.99×10^{-11}
Fibroblasts					
A (0.5–3.0)	2654.4 (S.D. 1425.5)	0.19*** (S.D. 0.09)	0.47*** (S.D. 0.28)	0.56*** (S.D. 0.66)	1.79*** (S.D. 1.13)
B (37–63)	4.60*** (S.D. 0.66)	0.85*** (S.D. 0.38)	3.20*** (S.D. 0.56)	2.63*** (S.D. 0.94)	3.95*** (S.D. 3.48)
C (177–250)	4.89*** (S.D. 0.47)	1.32* (S.D. 0.94)	3.48*** (S.D. 0.66)	2.91*** (S.D. 1.22)	4.79*** (S.D. 5.36)
D (420–841)	5.45** (S.D. 0.94)	1.22*** (S.D. 0.94)	4.79** (S.D. 0.94)	4.04*** (S.D. 1.79)	12.12*** (S.D. 11.56)
E Control	6.86 (S.D. 0.75)	2.07 (S.D. 0.47)	6.77 (S.D. 1.69)	12.40 (S.D. 1.79)	39.19 (S.D. 11.18)
P value	7.19×10^{-12}	6.77×10^{-9}	2.88×10^{-17}	7.66×10^{-23}	3.13×10^{-14}

All data were analysed by the analysis of variance (ANOVA) test. S.D. = standard deviation.
*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ when compared with control group.

Table 3 The changes in transforming growth factor-β1 (TGF-β1) concentration in culture medium for cells cultured with various-sized synthetic hydroxyapatite (HA) particles at a concentration of 1 mg ml^{-1} ($N = 10$)

HA particle size (μm)	TGF-β1 concentration (pg ml^{-1})				
	1 h	3 h	1 day	3 days	7 days
Myoblasts					
A (0.5–3.0)	1.8 (S.D. 0.9)	3.2 (S.D. 1.2)	9.9** (S.D. 3.3)	7.73*** (S.D. 12.3)	4.9*** (S.D. 6.5)
B (37–63)	1.4 (S.D. 0.3)	2.8 (S.D. 0.7)	20.8 (S.D. 8.3)	119.4 (S.D. 146.8)	100.1* (S.D. 37.2)
C (177–250)	1.3 (S.D. 0.2)	2.8 (S.D. 0.9)	24.4 (S.D. 7.9)	127.2 (S.D. 144.6)	99.8** (S.D. 21.8)
D (420–841)	1.7 (S.D. 0.6)	4.1 (S.D. 1.8)	21.9 (S.D. 6.5)	118.8 (S.D. 136.6)	94.6** (S.D. 25.5)
E Control	1.2 (S.D. 0.4)	2.7 (S.D. 1.7)	27.4 (S.D. 6.6)	128.3 (S.D. 134.7)	124.3 (S.D. 20.5)
P value	0.0671	0.1707	1.2×10^{-8}	9.2×10^{-12}	1.89×10^{-13}
Fibroblasts					
A (0.5–3.0)	1.1 (S.D. 0.3)	0.4 (S.D. 0.2)	7.988 (S.D. 3.1)	7.7*** (S.D. 3.9)	70.8*** (S.D. 35.5)
B (37–63)	1.8 (S.D. 1.5)	0.7 (S.D. 0.9)	16.5 (S.D. 6.1)	42.3 (S.D. 6.5)	302.7 (S.D. 64.3)
C (177–250)	1.9 (S.D. 2.1)	0.7 (S.D. 0.7)	18.1 (S.D. 5.3)	47.4 (S.D. 8.3)	335.2 (S.D. 67.4)
D (420–841)	1.7 (S.D. 1.3)	0.5 (S.D. 0.2)	21.3 (S.D. 5.7)	47.8 (S.D. 9.1)	349.1 (S.D. 56.4)
E Control	3.1 (S.D. 3.5)	0.5 (S.D. 0.2)	20.6 (S.D. 6.1)	40.4 (S.D. 8.9)	385.7 (S.D. 65.1)
P value	0.3347	0.8155	7.52×10^{-6}	9.25×10^{-16}	6.89×10^{-12}

All data were analysed by the analysis of variance (ANOVA) test. S.D. = standard deviation.
*$P < 0.05$; **$P < 0.005$; ***$P <$ 0.0005 when compared with control group.

changes of PGE_2 concentration in culture medium tested with the smallest particle size (0.5–3.0 μm) has a highest level and persisted longer (*Table 4*). The changes of PGE_2 concentration in culture medium corresponded to the changes of cell population when myoblasts were cultured with various sized HA particles. Similar results were observed with fibroblasts.

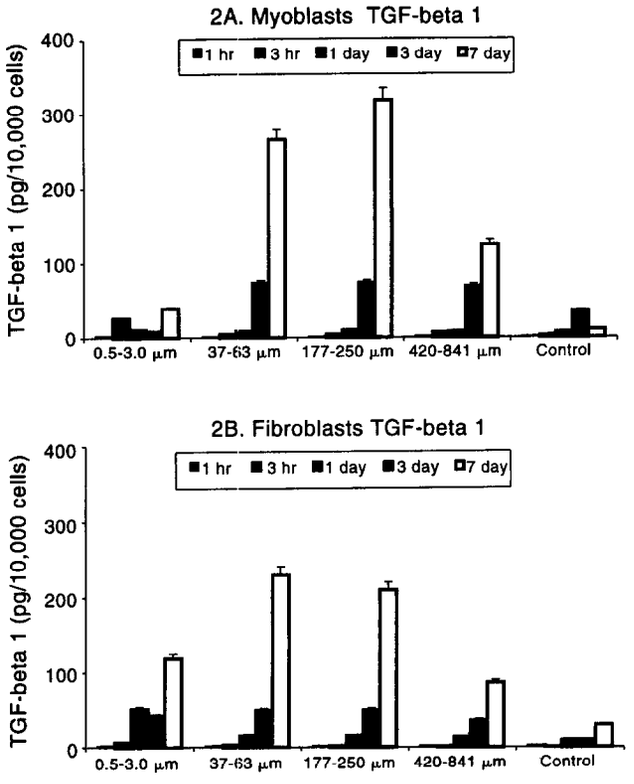

Figure 2 The changes in transforming growth factor-β1 (TGF-β1) titre for myoblasts (A) and fibroblasts (B) cultured with various-sized hydroxyapatite (HA) bioceramics (error bar = standard error). The TGF-β1 titre as a ratio to cell number was significantly affected by the addition of HA ($P < 0.005$). The TGF-β1 titre of all particle sizes increased as the culture time increased. The increase in TGF-β1 titre was most significant in the groups of medium-sized (37–63 and 177–250 μm) particles.

When calculated as a ratio to cell number, the PGE_2 titre in all particle sizes also significantly increased compared with the control. The changes in the PGE_2 titre were most significant in the groups tested with the smallest particle size (*Figure 3*). In the myoblasts, the PGE_2 titre reached its peaks at 3 h and 7 days after culture, while in the fibroblasts, the peak PGE_2 titre was observed on the first day of culture (*Figure 3*).

DISCUSSION

The revolution in biomaterials has promoted recent advancement in orthopaedic surgery. The events at the interface[13] between an implant material and the adjacent tissue are the direct result of the cellular, chemical, physiological and mechanical reactions evoked by the presence of the biomaterials[14]. In implantation, numerous cell populations and chemical factors are concerned. The *in vivo* experiments do not allow the examination of a specific cell to the substrate. In order to determine the sequences and the parameters influencing the interactive process, a model of cell culture in the presence of biomaterials is of great interest[15].

The ability of adult skeletal muscle fibres to regenerate after injury has been well documented since the eighteenth century[16]. Later, numerous investigators[17–22] reported that adult skeletal muscles did have great capability of regeneration in response to chemical and physical injury. The initial local tissue response to the implant is determined by the effect of normal bone healing in response to the trauma from implantation. However, the skeletal muscles and adjacent fibrous tissue were also exposed to the effect of implantation. The present study was designed to use the skeletal myoblasts and fibroblasts as a simple model for testing the effect of various sized HA particles on soft tissue cells.

Although cell-culture studies cannot directly duplicate the conditions that exist *in vivo*, the effects of specific types of particles on cellular metabolism can be directly quantified with this technique[23]. There

Table 4 The changes in prostaglandin E_2 (PGE_2) concentration in culture medium for cells cultured with various-sized synthetic hydroxyapatite (HA) particles at a concentration of 1 mg ml^{-1} ($N = 10$)

HA particle size (μm)	PGE_2 concentration (pg ml^{-1})				
	1 h	3 h	1 day	3 days	7 days
Myoblasts					
A (0.5–3.0)	828.1*** (S.D. 303.6)	801.1*** (S.D. 204.9)	1681.7*** (S.D. 59.9)	1828.7*** (S.D. 838.3)	2056*** (S.D. 332.5)
B (37–63)	115.6*** (S.D. 40.9)	94.9*** (S.D. 27.1)	669.9*** (S.D. 124.7)	1458.9*** (S.D. 787.1)	1704.1*** (S.D. 239.8)
C (177–250)	91.7*** (S.D. 29.7)	92.9*** (S.D. 19.7)	652.4*** (S.D. 111.1)	1401.7*** (S.D. 812.3)	1665.7*** (S.D. 251.3)
D (420–841)	162.6*** (S.D. 53.6)	74.8*** (S.D. 34.3)	481.6*** (S.D. 179.8)	1162.6*** (S.D. 850.9)	1206.9*** (S.D. 257.2)
E Control	21.4 (S.D. 4.2)	17.9 (S.D. 3.5)	76.1 (S.D. 42.2)	124.4 (S.D. 118.9)	28.7 (S.D. 6.3)
P value	6.75×10^{-17}	1.61×10^{-23}	9.49×10^{-31}	8.81×10^{-5}	3.48×10^{-22}
Fibroblasts					
A (0.5–3.0)	463.1*** (S.D. 155.9)	351.9*** (S.D. 68.9)	1574.6*** (S.D. 60.1)	1376.3*** (S.D. 133.1)	1707.2*** (S.D. 56.4)
B (37–63)	174.2*** (S.D. 44.3)	77.2*** (S.D. 13.6)	1046.6*** (S.D. 138.8)	987.9*** (S.D. 127.9)	1662.0*** (S.D. 56.8)
C (177–250)	171.4*** (S.D. 59.4)	74.2*** (S.D. 12.9)	1027.5*** (S.D. 122.5)	965.4*** (S.D. 199.9)	1650.2*** (S.D. 57.8)
D (420–841)	255.8*** (S.D. 109.3)	58.4*** (S.D. 13.6)	899.9*** (S.D. 321.4)	672.3*** (S.D. 173.4)	1176.2*** (S.D. 36.2)
E Control	44.9 (S.D. 9.5)	19.9 (S.D. 3.8)	451.1 (S.D. 359.5)	38.8 (S.D. 7.9)	83.1 (S.D. 11.2)
P value	9.85×10^{-12}	1.9×10^{-26}	3.92×10^{-12}	7.59×10^{-26}	1.01×10^{-25}

All data were analysed by the analysis of variance (ANOVA) test. S.D. = standard deviation.
*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$ when compared with control group.

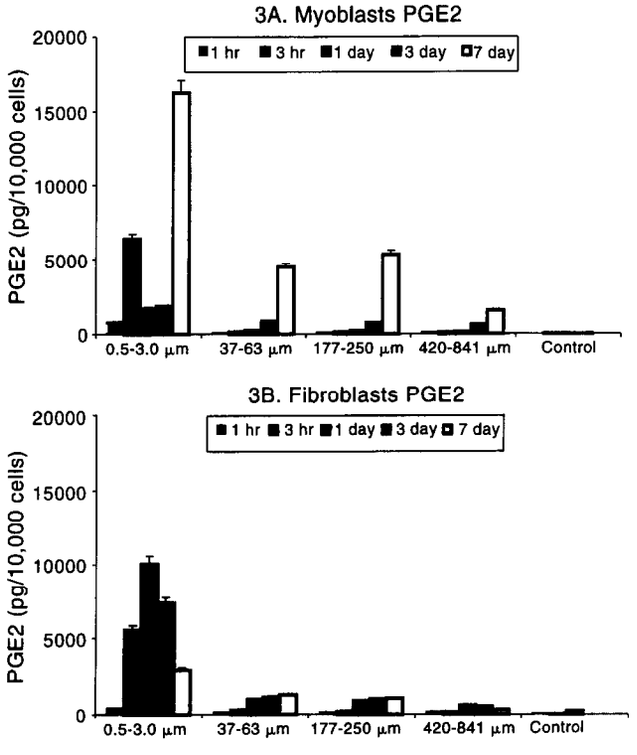

Figure 3 The changes in prostaglandin E_2 (PGE_2) titre when myoblasts (A) and fibroblasts (B) were cultured with various-sized hydroxyapatite (HA) bioceramics (error bar = standard error). When calculated as a ratio to cell number, the PGE_2 titre in all particle sizes increased significantly compared with that of the control ($P < 0.005$). The changes in the PGE_2 titre were most significant in the group tests with the smallest HA particle size. In the myoblasts, the PGE_2 titre reached its peaks at 3 h and 7 days after culture, while in the fibroblasts, the peak PGE_2 titre was observed on the first day of culture.

are several advantages of working with primary cell populations. Because these cells are not transformed, any observed response can be attributed to the phenotype of the cells and not as a possible by-product of the transforming process[24].

A decrease in cell number after exposure to the test material can be used as a marker for toxicity[25]. It was previously suggested that toxicity due to direct contact of particles with cells only occurs with particles smaller than about 5 μm in diameter[26]. In this study, adding HA particles into cell culture is found to significantly affect the cell count. When myoblasts and fibroblasts were cultured with hydroxyapatite particles, the cell populations of all experimental groups were significantly decreased. The cell population of the smallest sized hydroxyapatite has the fewest cell populations (*Table 1*). This is inconsistent with findings of Evans and Clarke-Smith[26]. The reduction in growth rate of the cells seen in this experiment could be due to an increase in the death rate of the cells or to a decrease in their mitotic rate.

It was reported that fine particles of hydroxyapatite, normally a very non-toxic material, could cause cell damage *in vitro*[27]. This toxicity depended on direct contact between cells and particles. The toxicity was associated with membrane damage[26]. There are statistically significant changes in the surface area of the cells after culture with HA particles. These differences were always statistically significant between the control group and various experimental groups (*Table 2*). This suggested that there were changes in the cell membrane after coculture with HA particles. Later, Evans[10] showed that the mechanism of the *in vitro* cell damage depends on a direct interaction between cells and particles and is largely independent of the chemical

nature of the particle. The invocation of a mechanism involving direct contact does not exclude toxicity due to other mechanism, such as leaching of toxic ions. However, the test material may have a low level of toxicity which, although not sufficient to kill cells, may inhibit normal cell function[25]. The effects of HA on the synthesis and secretion of specific proteins were also evaluated in this study.

The transforming growth factor-β1 has been shown to have stimulatory effects on cells[28,29]. In this study, TGF-β1 concentrations in culture medium were all significantly decreased by the addition of HA particles. The changes in TGF-β1 concentration in the medium with larger sized HA particles (i.e. 37–63, 177–250 and 420–841 μm) were quite similar and close to that of the control medium. The decrease in TGF-β1 concentration in the culture medium was most significant in the groups with the smallest particles (i.e. 0.5–3.0 μm HA) (*Table 3*). When calculated as a ratio to the cell number, the increase in the TGF-β1 titre was most significant in the groups of medium-sized (i.e. 37–63 and 177–250 μm) particles (*Figure 2*). This may suggest that the medium-sized HA is more compatible than that of small particles, as the autoinductive processes can amplify and extend the activity of TGF-β1[29].

For a given mass of bone cement, smaller particles (less than 20 μm) resulted in more inflammation than large particles (50–350 μm)[30]. Irregularly shaped particles produced a greater response than spherical particles. Large particles induced a more intense rise in the white blood-cell count and in the production of prostaglandin E_2[30]. In this study, the PGE_2 concentrations in the experimental medium were always significantly higher than that of the control medium. The PGE_2 concentration in the control medium increased from day 1 to day 3, then rapidly decreased to 7 days culture (*Table 4*). When cells were cultured with HA particles, the PGE_2 concentration reached its peak gradually to day 7. If we calculated this as a ratio to cell number, the PGE_2 titre in all particle sizes also significantly increased compared with the control. The changes in the PGE_2 titre were most significant in the groups tested with the smallest particle size (*Figure 3*).

Prostaglandin E_2 is a known regulator of protein turnover in skeletal muscle[31]. Prostaglandin E_2 stimulates muscle protein degradation[32,33]. In this study, the changes in PGE_2 concentration in the culture medium were closely related to the changes in cell population when myoblasts were cultured with the various sized HA particles. The inhibitory effects of the HA particles on the cell culture were possibly mediated by the increased synthesis of PGE_2[34]. It is reported that the ability of various cytokines (including interleukin-1, tumour necrotic factor and transforming growth factor-α) to stimulate bone resorption is mediated by increased PGE_2 synthesis and production of these cytokines is influenced by prostaglandins[34].

The HA supplied for clinical use is larger than the largest used in this experiment. The results on the effect of various sized HA particles on myoblasts do not mean that the same result will occur in the block form. It does manifest the *in vivo* effect of the degradation product of HA on the adjacent ingrowing myoblasts. They suggest that caution should be exercised before considering the use of HA products which could easily break down into a fine powder, however well tolerated the bulk form is. In this work, only the biocompatibility of various sized HA particles to the myoblasts and fibroblasts was elucidated. Further studies on the relationships of various sized HA particles to leucocytes are now in progress.

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