Hydroxyapatite coated with insulin-like growth factor 1 (IGF1) stimulates human osteoblast activity in vitro

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We studied the effects of a hydroxyapatite-tricalcium phosphate material coated with Insulin-like Growth Factor 1 (IGF1) on cell growth, collagen synthesis and alkaline phosphatase activity (ALP) of human osteoblasts in vitro. Cell proliferation was stimulated when osteoblasts were incubated with untreated hydroxyapatite (HA) and it was further increased by exposure to IGF1-coated HA. ^{3}H-Proline uptake was significantly increased by treatment with either HA or IGF1-coated HA but no significant differences were found between these two groups. ALP activity was enhanced by exposure to HA, with respect to the control, and further increased by treatment with IGF1-coated HA. Our findings suggest that HA is useful for promoting osteoblast activity and IGF1 may help to improve its biological characteristics.

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Hydroxyapatite (HA) is commonly used in orthopedic surgery to improve bone fixation of uncemented joint prostheses (Söballe and Övergaard 1996) and to fill bone defects (Oonishi et al. 1997), and it is known to enhance bone formation (Capello 1994, D'Antonio et al. 1996, Munting 1996). In histologic analysis of HA-coated acetabular components retrieved at autopsy (Bauer et al. 1993), a better bone apposition was documented in the HA-coated implants than in the uncoated controls. In this study, we used an in vitro experimental system to evaluate the effects of a commercial HA-tricalcium phosphate material, coated or not with Insulin-like Growth Factor 1 (IGF1), on several parameters of human osteoblast activity. It has already been shown that IGF1 strongly stimulates either bone collagen synthesis or osteoblast cell lineage replication (Canalis et al. 1991). It has also been found to be chemotactic for human osteoblasts, even if less effective than other growth factors (TGF-ß, PDGF-BB) (Lind et al. 1995). All these properties could be useful for improving implant fixation in the early postoperative period. The cellular parameters evaluated were 1) cell proliferation, 2) collagen synthesis and 3) alkaline phosphatase activity.

Material and methods

Biomaterial

We used the biomaterial Triosite (Zimmer, Swindom, U.K.). This is a porous biphasic material composed of 65% SE 15 hydroxyapatite and 35% SE 15 tricalcium phosphate, with less than 15% of other phosphates. The Ca/P ratio is 2, SE 0.2 and the pore size is 400 µm, SE 150. It was crushed into 0.5 mm^3 particles, sterilized in alcohol, coated with IGF1 (Eli Lilly & Company, Indianapolis, USA) and used for experiments. IGF1 was added to the HA by dipping the granules in a solution of 10^{-8} M of IGF1 at 4 °C for 24 hours. Then HA coated with IGF1 was air-dried at room temperature on sterile gauze and transferred to culture dishes for the experiments.

Bone marrow-derived osteoblast preparation

Surgical specimens of human bone marrow tissue were obtained within 1 hour of total hip arthroplasty. Marrow cells were harvested by washing with α-MEM (alpha-Minimum Essential Medium, Gibco Ltd., Uxbridge, U.K.) and pelleted by centrifugation at 500 g for 5 minutes at 4 °C. The cell pellet was resuspended in 10 mL α-MEM and passed through a nylon mesh (90 µm pore size). Cells were plated in 25

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cm^2 flasks (Falcon Becton-Dickinson, Lincoln Park, NJ) and cultured in α-MEM, supplemented with 10% FCS (fetal calf serum, Gibco Ltd., Uxbridge, U.K.), 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B and 50 IU/mL mycostatin (Eurobio, Paris, France) at 37 °C, in a water-saturated atmosphere containing 5% CO_2. Media were changed every 3 days.

Osteoblast characterization

After 48 hours, osteoblasts were characterized according to well-established parameters of osteoblastic phenotype: alkaline phosphatase activity, cAMP production in response to 10^{-8} mol/L PTH and osteocalcin synthesis, in response to 10^{-8} mol/L 1,25-dihydroxy-vitamin D_3. Results (not shown) demonstrated that the cells used for the experiments were well-differentiated osteoblasts. The cells were all utilized between passages 6 and 9.

Cell proliferation

Proliferation assays were performed in 96-well microtiter plates using serum-free α-MEM supplemented with 0.5% BSA (fatty acid-free bovine serum albumin, Sigma Chemical Co., St. Louis, MO). In all the experiments, cells were exposed for 48 hours to HA or to IGF1-coated HA (HA-IGF1). A control group was studied, culturing the cells without HA for 48 hours. After that period, osteoblasts were gently washed with PBS (phosphate buffer saline), dehydrated with 20% methanol for 10 minutes and stained with 0.5 % crystal violet for 5 minutes, followed by extensive rinsing. The dye was released from the cells by the addition of 0.1 M Na citrate in 50% ethanol. The optical density of the stain solution released was read in a Titertek colorimeter at 540 nm wavelength.

Incorporation of ^{3}H-Proline

Collagen synthesis was assessed with ^{3}H-Proline (Amersham International, Amersham, U.K.) uptake. All the experiments were performed with serum-free α-MEM, supplemented with 0.5% BSA. Cells were cultured with HA or HA-IGF for 48 hours while, in the control group cells were cultured without HA for 48 hours. After 44 hours, 10 μL of α-MEM containing 5 μCi of ^{3}H-Proline was added to each experimental well and incubated for a further 4 hours. A short pulse-time was necessary to avoid cell secretion of ^{3}H-hydroxyproline with tropocollagen fibers. Osteoblasts were then washed with PBS, solubilized in 0.5 mL of 0.1% SDS (sodium dodecyl sulfate) and precipitated by addition of 100% TCA (trichloroacetic acid). After 30 min of incubation at 4 °C, TCA-precipitable material was pelleted by centrifugation at 955 g, redissolved in 500 μL of 0.1% SDS and counted in a Beckman 6000 scintillation spectrophotometer, after addition of 500 μL of distilled water and 9 mL of scintillation fluid (Beckman Instruments Inc., Fullerton, CA). Results were expressed in counts per minute.

Alkaline phosphatase activity

To test alkaline phosphatase activity, cells were incubated for 48 hours with HA or HA-IGF1 in serum-free α-MEM supplemented with 0.5% BSA. A control group was studied, culturing the cells without HA for 48 hours. Then osteoblasts were solubilized in 0.5 mL of 0.1% SDS and the cell lysate was incubated for 2 hours in the presence of the substrate (p-nitrophenol phosphate) at 37 °C. Samples were read in a spectrophotometer (analytical wavelength: 410 nm) and results expressed in ng/μg of cell proteins.

Statistics

All the experiments were performed in quadruplicate and repeated three times. A one-way analysis of variance (ANOVA) was used to determine any significant difference between the various treatments. When significance was found, a Newman-Keuls multiple comparison test was used to establish where difference occurred. Significance was set at $p < 0.05$.

Results (Table)

Cell proliferation

HA significantly stimulated cell proliferation with respect to untreated control wells. Treatment with HA-IGF resulted in a further and significant increase in proliferation with respect to the control and HA groups.

Collagen synthesis

HA and HA-IGF treatment stimulated ^{3}H-Proline uptake with respect to the control, but there was no difference between the two groups.

Alkaline phosphatase activity

Treatment with HA induced increased alkaline phosphatase activity by the osteoblasts. In cells treated with HA-IGF, alkaline phosphatase activity was further increased. The differences between the 3 treatments were all significant.

Discussion

The rationale for HA and tricalcium phosphate use in

Effect of hydroxyapatite (HA) and of hydroxyapatite coated with IGF1 (HA-IGF1) on human osteoblast proliferation, collagen synthesis and alkaline phosphatase activity. Results (SD) are expressed as the mean of 3 experiments performed in quadruplicate

Treatment	Control n 12	HA n 12	HA-IGF1 n 12
Millilabsorbance unit at 540 nm	513 (57)	725 (82) [a]	838 (44) [a,b]
Counts per minute	6856 (1236)	12598 (3512) [a]	14815 (4384) [a]
Alkaline phosphatase activity, ng/µg proteins	3.25 (1.7)	7.75 (3.2) [a]	18.7 (2.8) [a,b]

[a] $p < 0.05$ versus control
[b] $p < 0.05$ versus HA
Since the ANOVA test was significant ($p = 0.02–0.001$), we used the Newman-Keuls multiple comparison test.

orthopedic surgery is their ability to improve bone formation and therefore they are extensively used to fill bone defects (Oonishi et al. 1997) and to coat joint prosthesis (Söballe and Övergaard 1996). In our study, a commercial HA-tricalcium phosphate material (Triosite) was coated with IGF1 to see whether its biological characteristics could be improved. Various HA coatings have been made with bone morphogenetic proteins, bFGF and TGF-ß, and tested in vivo on animals, the results being evaluated by histology, histomorphometry, mechanical strength tests, x-ray films and dual x-ray densitometry (Suwa et al. 1993, Ono et al. 1995, Lind et al. 1996). In this study, we performed in vitro experiments to investigate several metabolic and biochemical cellular parameters of osteoblast activity, using HA and IGF1-coated HA. We chose IGF-1 because, although less effective than other growth factors, it has a well demonstrated autocrine-paracrine activity on osteoblasts (Canalis et al. 1991). HA itself significantly stimulated the osteoblast proliferation rate with respect to the control, while cell replication in the presence of HA-IGF1 reached even higher values (Table). These results suggest that HA can stimulate cell replication and that IGF1 can improve it. An increased cell proliferation could signify an osteoblast de-differentiation, but since alkaline phosphatase activity was increased in both the HA and HA-IGF1 experiments (Table), osteoblast de-differentiation is unlikely. In fact, an increase of this enzyme activity is typical of well-differentiated osteoblasts (Fedarko et al. 1990). After exposure to HA, osteoblasts increased collagen synthesis. This effect has also been found in clinical studies (Furlong and Osborn 1991), and it explains the effectiveness of HA in improving bone formation.

Alkaline phosphatase activity was greatly increased after HA treatment, with respect to the control (Table), and its activity was enhanced by IGF-1 treatment. These results indicate that HA is effective in stimulating bone matrix calcification, since alkaline phosphatase is an enzyme of capital importance for this event to occur and the coating with IGF1 further improved this process.

Clinical applications of HA coated with IGF1 or other growth factors (BMPs, TGF-ß, bFGF) may be useful for filling bone defects in which it is advisable to have both good cell proliferation and bone formation. Its use in joint replacement surgery might be a problem, because of the difficulty in coating the prosthesis with growth factors, without risk of contamination.

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