The interactions of bisphosphonates in solution and as coatings on hydroxyapatite with osteoblasts

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Aseptic loosening is one of the major causes of failure of artificial hip joints, and it can occur for several reasons, including osteolysis of the bone tissue in response to stress shielding or cellular reactions to wear debris. Any treatment of the prosthesis which could minimize the osteolytic response of bone tissue may be able to extend the life-time of the implant. Bisphosphonates are potent inhibitors of osteoclastic bone resorption, and they bind avidly to hydroxyapatite (HA). Coating the prostheses with bisphosphonates may therefore inhibit osteolysis. We have investigated the potential for this approach by determining whether bisphosphonates interact with osteoblasts *in vitro*. The effects of pamidronate (P), clodronate (C), and etidronate (E) in solution and when coated onto HA were investigated. P inhibited protein and collagen syntheses potently when in solution, but not after being bound to HA. When bound to HA, both P and C increased DNA, protein and collagen syntheses of osteoblasts and may encourage the osseointegration of implants. The pharmacological effects of the bisphosphonates studied altered dramatically after binding to HA. This must be fully investigated before this approach to prolonging prostheses stability can be evaluated.

Introduction

There are currently around 500 000 artificial hips implanted annually worldwide [1], and increasing numbers of these are being implanted into younger patients. The life expectancy of artificial hips is limited, and few survive beyond 20 years. Thus, the need to develop artificial hip joints with extended life-times has become an important issue.

The most common cause of failure is aseptic loosening where, in the absence of infection, after a certain amount of wear, the fixation of the implant into the bone fails, and the artificial joint becomes loose. Aseptic loosening may occur for several reasons, including osteolysis of the bone tissue in response to stress shielding, osteolysis caused by cellular reactions to wear debris, and/or motion at the bone–cement–implant interfaces [2]. Factors such as surgical technique, initial stability and mechanical factors will also contribute. Any treatment of the prostheses which could miminize the osteolytic response of the bone tissue in response to both stress shielding or wear debris may be able to extend the life-time of the implant.

Bone remodeling occurs continuously in the skeleton by the co-ordinated actions of osteoblasts, which secrete new matrix, and osteoclasts, which resorb old bone. Any agent which reduces the efficiency of the later process will inhibit the osteolysis associated with prosthesis loosening. Bisphosphonates are the most effective inhibitors of osteoclastic bone resorption [3–10]. They are pyrophosphate analogs in which the oxygen in the P–O–P has been replaced by a carbon to yield a P–C–P backbone. Substitutions on the carbon yield a large family of compounds with different properties and potencies which are determined by nature of the side chains [11]. Bisphosphonates are used therapeutically in a variety of diseases of enhanced bone resorption, including Paget's disease, hypercalcemia of malignancy, and osteoporosis, so there are data available on their

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efficacy, safety and pharmacodynamics in humans following both oral and intravenous administration [11]. Studies have suggested that the mechanism by which these compounds inhibit bone resorption is at a cellular level, affecting cells of the osteoclast lineage [3–10]. They have two modes of action on osteoclastic bone resorption, inhibiting both the acession (migration to the bone surface and fusion) of osteoclast precursors, and resorption by the mature osteoclast [3, 6, 8–10]. The latter occurs as a result of the bisphosphonates being internalized into osteoclasts by fluid-phase pinocytosis or by phagocytosis [9] and, once internalized, they affect a multitude of biochemical processes such as cell number, production of lactic acid [4], disruption of the osteoclast cytoskeleton [9], and prostaglandin production [7]. These result in a loss of the ability to resorb bone or even osteoclast cell death.

Bisphosphonates bind avidly to the bone mineral hydroxyapatite (HA), and in humans the drugs are released only slowly during skeletal remodeling. For example, the half-life of alendronate in humans is approximately 10 years [12]. Previous reports have shown that bisphosphonates may be immobilized on HA-modified titanium for dental applications [13]. We propose that a coating of bisphosphonate on the HA ceramic of artificial hip joints may function to inhibit osteolysis of the surrounding tissue, reduce the tendency towards loosening and, therefore, improve the life-time of the implant. However, it should be borne in mind that many of the bisphosphonates also inhibit mineralization, albeit at concentrations up to 1000-fold higher than those which inhibit resorption. The relative potency varies tremendously within the bisphosphonate drugs. Etidronate, for example, inhibits mineralization at doses very close to those which inhibit resorption [14], and it has been shown to inhibit the calcification of bioprosthetic heart valves. To take advantage of this property investigations are underway to bind bisphosphonates covalently to the valves, so there is a precedent for coating medical devices with bisphosphonates. To determine whether this approach is feasible for orthopedic implants, there are several questions which need to be addressed. In the first study aimed to answer these questions we have investigated the effect of bisphosphonates, both in solution and when coated onto HA material, on the viability, growth and function of osteoblasts. There is little information available on the interaction of bisphosphonates with osteoblasts. However, what is known, is that bisphosphonates have various inhibitory and stimulatory affects on the secretion and proliferation of osteoblasts which differ from one species to another [15–18]. In cultured human osteoblasts etidronate and pamidronate stimulate mineralization at concentrations below 10 nM, but are inhibitory above 1 μM [18]. ^{3}H-Thymidine uptake into DNA is also inhibited by both of these drugs (10 nM) [17], and in addition, Igarashi *et al*. [16] found that bisphosphonates inhibited the proliferation of mouse osteoblasts at concentrations of 250 μM. Thus, although bisphosphonates have well-documented effects on bone resorption, their effects on bone formation may also be important. Although recent studies have shown that pamidronate immobilized on HA-coated titanium surfaces does not have any toxic effect on osteoblastic cells [13] further consideration of the effects on the bone mineralization process is required. Our study, therefore, aimed to determine whether the drugs could interfere with the formation of bone matrix when coated onto implants and thereby impede osseointegration.

The interaction with osteoblasts was studied using an immortalized rat osteoblast cell line generated in our laboratory by transfection of SV40 DNA into primary calvaria cells. These cells (FFC cells) have been used extensively for biocompatibility studies of several orthopedic materials [19–21], and their responses compare favorably with those of primary cells.

Materials and methods

Preparation of materials

Sodium clodronate ((C), Bonefos, Boehringer Ingelheim, Bracknell, Berkshire, UK), Etidronate ((E), Didronel, Procter & Gamble, Staines, UK) and Pamidronate ((P), Aredia, Ciba, Camberley, Surrey) were prepared as 0.22 M, 0.049 M, and 0.038 M solutions in serum-free medium respectively. The structures of these drugs are shown in Fig. 1.

To produce pellets of dense HA material (pore sizes < 3 μm) 55 g of calcium phosphate powder (tribasic 34–4% Ca) was mixed with 10 ml of 4% (w/v) polyvinyl alcohol in distilled water. Using an Instron machine 1 g of powder was placed in a compact motor and pressure of 750 kg applied to produce 1 cm × 0.5 cm dense pellets. The samples were then sintered at 1150 °C for 2 h. After sintering, samples were polished using silicone carbide paper, and then cleaned for 1 h in 70% (v/v) alcohol, and then for a further 1 h in distilled water at 37 °C. A number of polished and cleaned samples of the material were then coated with 4 × 200 μl aliquots of either 3 mg/ml disodium clodronate or 1 mg/ml disodium pamidronate. The coated substrates were dried under a controlled,

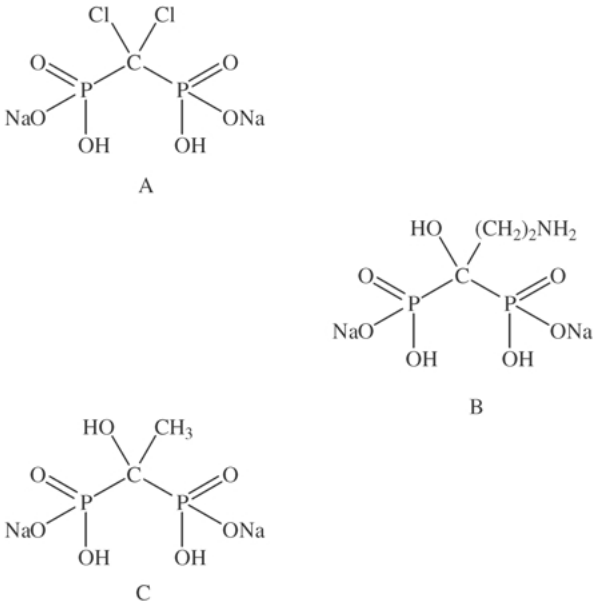

Figure 1 Chemical structures of the sodium salts of (A) clodronate, (B) pamidronate and (C) etidronate.

constant heated airflow between each application. The presence of the coating was confirmed using Joel JSM 6310 scanning electron microscope with an Oxford ISIS energy dispersive X-ray (EDX) microanalysis attachment using an accelerating voltage of 20 kV.

Cell culture and treatment of cells

Immortalized rat osteoblasts (FFC cells) were generated by transfection of primary osteoblasts isolated from rat calvaria as described previously (Macnair *et al*. 1997). They were routinely grown in Dulbecco's modification of Eagle's medium (DMEM) containing 10% v/v foetal calf serum (FCS). The FCS was from Seralab and DMEM was supplied by Gibco BRL Life Technology. P was added to the cells in calcium-free medium, which was also supplied by Gibco BRL Life Technology, as it precipitates in the presence of calcium. For collagen synthesis (by incorporation of tritiated proline), DNA, RNA and protein synthesis, measurements of protein and reduced glutathione (GSH) content and the MTT assay cells were seeded at a density of 2×10^4 cells/cm^2 in 24 well plates or on 1 cm diameter disks of the HA ceramic materials. For detecting the presence and distribution of collagen and actin in the cultures by immunochemical techniques using confocal laser scanning microscopy (CLSM) the cells were seeded at 2.5×10^3 per cm^2. The coated and uncoated samples of HA ceramics were sterilized overnight by UV light and then 100 µl of cell suspension was added to the surfaces of the materials in 24 well plates for 1 h to allow attachment. After the attachment period, 1 ml of medium was added to cover the samples completely and they were incubated at 37 °C in an atmosphere of air/5% CO_2.

Determination of cell functions

The functions of the cells were determined after adding the bisphosphonates to the medium of cultures, allowing 24 h for the cells to adhere to the culture dishes before addition of the drugs. Cells were exposed to 1 mM of each drug for 48 h. Functions were also tested after culturing the osteoblasts on uncoated and bisphosphonate coated dense HA ceramic for 3–4 days as indicated in the results section. Again cells were allowed to adhere to these materials before addition of the drugs.

To measure the affect on cell growth rate (assessed by total cell protein) and intracellular GSH levels, after 48 h culture for the drugs in solution, and 4 days for the cells cultured on the ceramic, cultures were washed with phosphate buffered saline (PBS) and intracellular GSH extracted with 10% (w/v) trichloroacetic acid (TCA) for 10 min at room temperature. Extracts were stored at − 20 °C until analysis of the GSH content by the fluorimetric assay described by Hissin and Hilf [22]. Total cell protein was determined by the method of Lowry *et al*. [23] after digestion of the cells with 0.5 M NaOH for 18 h at 37 °C.

The interaction of the bisphosphonates with the cells was also assessed using the MTT assay. For the drugs in solution the MTT test was carried out in 96 well plates using 1 mM of each drug and exposing the cells to the drugs for 48 h. The method used was that described by Macnair *et al*. [7] for FFC cells. For the ceramic samples, the MTT test was carried out in 24 well plates. Following a culture period of 3 days on the ceramics, 0.5 ml of 10 mM MTT in PBS, pH 6.75, was added to each well. Reduction of the MTT was allowed to proceed for 4 h, then the formazan product was dissolved in 1 ml dimethylsulfoxide (DMSO). This solution was transferred to a cuvette, and the optical density measured in a Shimadzu UV-2101 spectrophotometer at 540 nm.

The effects on RNA, DNA and protein synthesis were measured following exposure to 1.0 mM of each drug for 24 h (after allowing 24 h for cells to adhere) in the presence of 7.4 kBq of either 5,6-^{3}H-uridine (1.63 TBq/m mol), methyl-^{3}H-thymidine (3.03 TBq/m mol) or L-4,5-^{3}H-leucine (5.62 TBq/m mol). All isotopes were supplied by Amersham Life Science. Osteoblasts cultured on the uncoated and coated ceramic were also incubated with the isotopes and drugs for 24 h, after allowing 24 h for the cells to adhere, and syntheses of the macromolecules measured by the same procedures for both experiments. Following the 24 h period for incorporation of the radioactivity the cell samples were processed as described previously [24].

Collagen synthesis was assessed both by incorporation of ^{3}H proline, and by immunocytochemical staining. For measuring the effect on incorporation of ^{3}H-proline, after allowing 24 h for cells to adhere, they were exposed to 1.0 mM of each drug in solution for 24 h in the presence of 18.5 kBq L-2,3-^{3}H-proline (1.4 TBq/m mol, NEN Products, Boston, MA). The osteoblasts cultured on the uncoated and bisphosphonate coated ceramic were also incubated with the drugs and radiolabeled proline for 24 h, after allowing 24 h for cell adhesion to the surfaces, and the same procedure was followed for both experiments. After the 24 h incubation with ^{3}H-proline, the cells were washed with 1 mM ''cold'' proline in PBS for 10 min at room temperature to remove non-specific binding, and digested in 2 ml 0.5 M acetic acid overnight. 0.5 ml samples were neutralized with 0.5 M NaOH, then 20 µl of 50 mg/ml (400 units) Type IV collagenase (Sigma Chemical Co.) in Hank's Balanced Salt Solution, pH 7.4, was added and the cells incubated for 1 h at 37 °C. The digestion was stopped by adding 0.1 ml of 10% (w/v) TCA, and, after centrifuging at 13 000 rpm for 10 min, the supernatant was mixed with 4 ml scintillant and used to measure ^{3}H-collagen formation by scintillation counting.

Secretion and distribution of collagen in the presence of the bisphosphonates in solution was examined after 3 days exposure to 1 mM of each drug. Immunocytochemical studies were not carried out on the ceramic samples because the avidin-fluorescein isothiocyanate (FITC) bound strongly to the material itself and prevented visualization of the collagen. After being washed with PBS, then incubated with 0.1 mg/ml avidin in PBS for 2 h at 37 °C, and fixed in 4% formalin in PBS (30 min at 37 °C, followed by 3 PBS washes over a period of 2 h), the cells were incubated with 2% (w/v) casein in PBS for 30 min at 37 °C. Cells were then washed a further 3 times with PBS, and incubated with type I collagen-biotin antibody (Southern Biotechnology Associates, Inc, USA) for 18 h at 4 °C to label the collagen, before staining for 3 h at 37 °C with avidin-

FITC. Examination by CLSM took place immediately after sample preparation.

The affect of the drugs on the distribution of actin in the cells was investigated after 3 days exposure to 1 mM of each drug. Cells were fixed, and washed with PBS as described above. Then they were incubated with FITC-conjugated phalloidin (Sigma Chemical Co) in PBS for 60 min at 37 °C to label the actin. Samples were immediately examined by CLSM.

CLSM examination used 488 nm excitation with a 510 nm dichroic beam splitter in place. Data from the two channels were collected, using a barrier filter of 590 nm in channel 1, and a band pass filter of 530 nm in channel 2. The objective lens was × 40 oil immersion/1.3 NA.

Results

Fig. 2 shows the EDX spectra for the uncoated and coated HA disks. The presence of chlorine in Fig. 2(b) and the enhanced carbon peak in Fig. 2(c) confirms the presence of clodronate and pamidronate on the surface of the coated HA disks respectively.

Fig. 3(a) shows the affect of C, E and P on cell growth in terms of protein content when added at a concentration of 1 mM to the cell culture medium. P was added in calcium-free medium as it precipitated in the presence of calcium. Control cultures were set up in the calcium-free medium and these should be used to assess the effects of this drug. After 48 h exposure only P had any significant effect on cell protein content, causing a significant decrease when compared with the cells cultured in the control calcium-free medium. In contrast, neither C nor E had a significant effect on cell protein content.

The HA material was coated with C and P from solutions of 3 mg/ml and 1 mg/ml respectively. There was no significant alteration in cell growth in terms of protein content when the cells were cultured on the uncoated material compared with the polystyrene control. Furthermore, coating the HA material with either C or P had no significant effect on cell growth (Fig. 3(b)).

GSH content was used to monitor the redox balance of the cells and as an index of viability. The drugs had no significant effect on GSH content of the cells either in solution, or when coated onto the HA material (results not shown). The GSH content of control FFC cells grown on polystyrene for 48 h was 14 pmol/mg protein.

The cell growth rate and viability in the presence of the drugs was further evaluated by the MTT assay which reflects the activity of intracellular reductases and the availability of cofactors NADH and NADPH required for their activity. The results show that there was a significant decrease in the reduction of MTT when C was added to the medium (Fig. 4(a)). There was no effect on cellular MTT activity of either C or P when the HA material was coated with the drugs (Fig. 4(b)).

Macromolecular synthesis, including synthesis of RNA, DNA, protein and collagen, was measured after 24 h exposure to 1.0 mM for each drug and is shown on Figs 5 and 6. When added to the medium there was no affect of the drugs on RNA synthesis (Fig. 5(a)). Under the same conditions, E inhibited DNA synthesis, (decreased to 44% of control cell levels) and P was

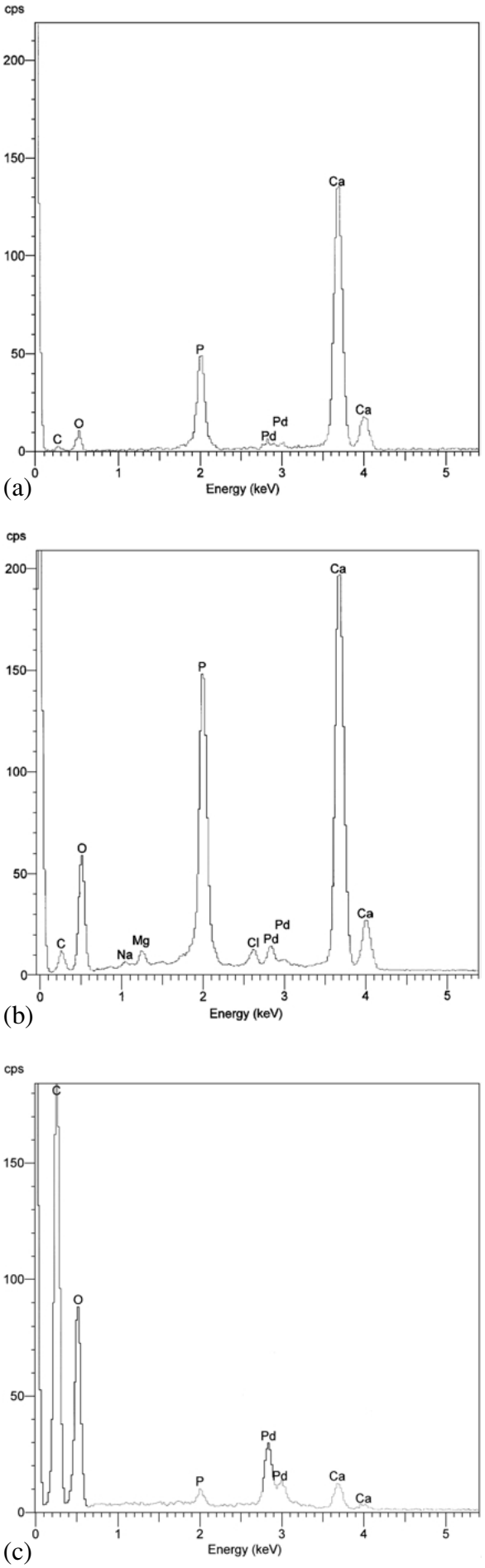

Figure 2 EDX microanalysis spectra for uncoated (a), clodronate-coated (b) and pamidronate-coated (c) HA disks obtained using a Joel JSM 6310 scanning electron microscope with an Oxford ISIS EDX system at an accelerating voltage of 20 kV.

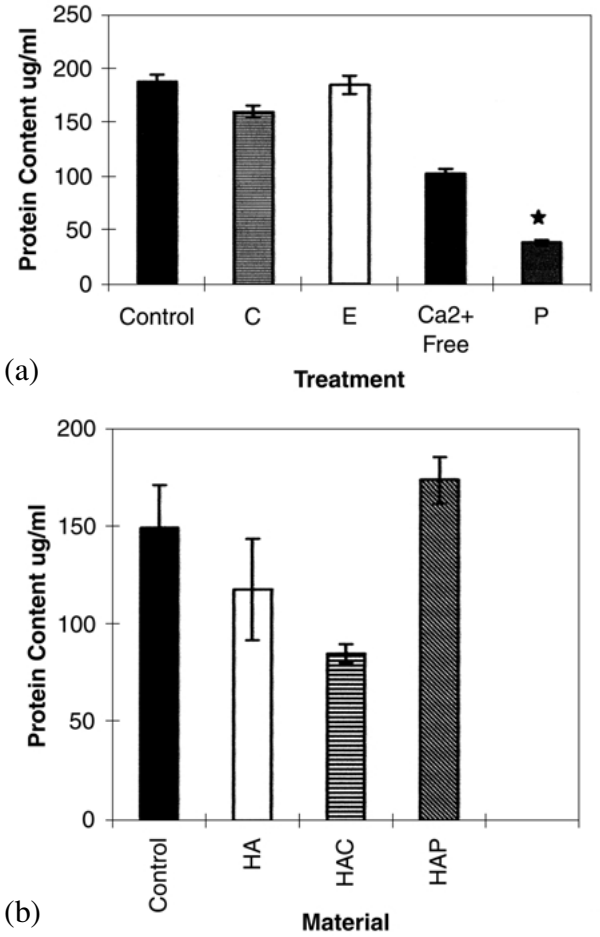

Figure 3 Protein content of FFC cells after exposure to the bisphosphonates. (a) shows the effect of clodronate (C), etidronate (E) and pamidronate (P) when added to the cell culture medium for 48 h. The drugs (1 mM) were added 24 h after the cells were seeded. P was added in calcium-free medium, and a control was included with cells grown in this medium in the absence of P. Results are means $\pm$ SEM, of six experiments. $^*P < 0.05$, compared with appropriate medium controls. (b) shows the growth of cells on polystyrene control dishes, uncoated hydroxyapatite (HA), and HA coated with either clodronate (HAC) or pamidronate (HAP) for 4 days. Results are means $\pm$ SEM, of five experiments.

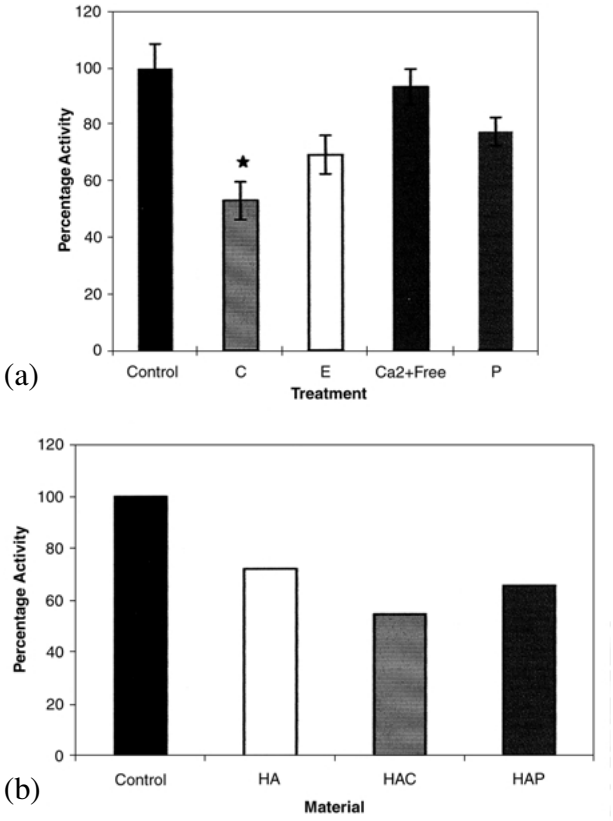

Figure 4 MTT activity of FFC cells after exposure to the bisphosphonates. (a) shows the effect of clodronate (c), etidronate (e) and pamidronate (P) when added to the medium for 48 h. The drugs (1 mM) were added 24 h after the cells were seeded. P was added in calcium-free medium, and a control was included with cells grown in this medium in the absence of P. Results are means $\pm$ SEM of 16 experiments. $^*P < 0.05$, compared with appropriate medium controls. (b) shows the MTT activity of cells grown on polystyrene control dishes, uncoated hydroxyapatite (HA), and HA coated with either clodronate (HAC) or pamidronate (HAP) for 3 days.

found to inhibit both protein (decreased to 29% of control cell levels) and collagen (decreased to 19% of control cell levels) synthesis (Fig. 6(a)) significantly. This inhibitory effect on protein synthesis was not observed when the HA material was coated with P. Results for the coated samples (Figs 5(b) and 6(b)) show that there was a significant increase in DNA, protein and collagen synthesis in cells cultured on surfaces coated with either C or P. For example, cells grown on control HA samples incorporated 6570 ± 821 dpm proline into collagen whereas cells grown on HA samples coated with C and P incorporated 16467 ± 2743 and 13764 ± 3236 dpm, respectively.

Control cells grown on glass coverslips for 3 days and stained with FITC-phalloidin before examination by CLSM clearly showed the distribution of actin microfilaments within FFC cells (Fig. 7(A)). Long fibers can be seen aligned in parallel throughout the length of the cells. Exposure of FFC cells to 1 mM of C or P in the medium (Fig. 7(B) and (D)) did not appear to affect the distribution of actin microfilaments. However, cells exposed to E particularly seemed to contain areas of highly fluorescent, disorganized actin staining (Fig. 7(C)).

Assessment of collagen production in control cells by immunostaining showed the appearance of collagen fibers as a layer over the cells (Fig. 8(A)). The cells exposed to C and E produced a similar layer of fibers to that observed in the control cell cultures (Fig. 8(B) and (C)). However, there was a dramatic affect when cells exposed were exposed to P (Fig. 8(D)); few collagen fibers were produced. This confirms the findings on Figs 5(a) and 6(a), where P in solution was also shown to significantly inhibit protein synthesis and collagen synthesis even after only 24 h exposure, as measured by incorporation of radioactive precursors.

Discussion

This study has been conducted using relatively high concentrations of the drugs involved. Serum concentrations of these bisphosphonates in patients undergoing therapy for Paget's disease or osteoporosis are in the range $0.25–6 \times 10^{-5}$ M/kg body mass [11]. However, the concentrations used in this study reflect the high local concentrations that might be experienced by the cells surrounding a coated implant.

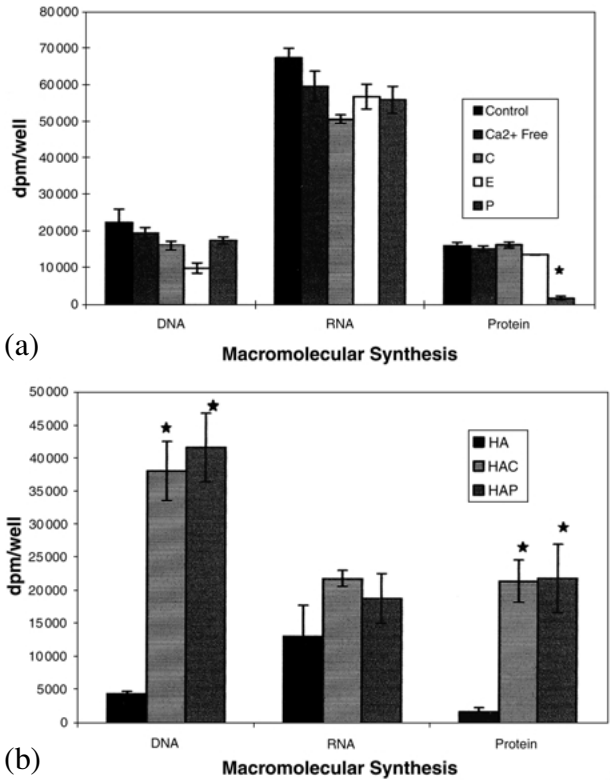

Figure 5 DNA, RNA and protein syntheses in FFC cells after exposure to the bisphosphonates. (a) shows the effect of clodronate (C), etidronate (E) and pamidronate (P) when added to the cell culture medium for 24 h. The drugs (1 mM) were added 24 h after seeding the cells. P was added in calcium-free medium, and controls were included with cells grown in this medium without P. (b) shows the macromolecular syntheses in cells grown on uncoated hydroxyapatite (HA), and HA coated with either clodronate (HAC) or pamidronate (HAP). Results are means ± SEM, of four experiments. $*P < 0.05$, compared with medium controls (A) or HA controls (B).

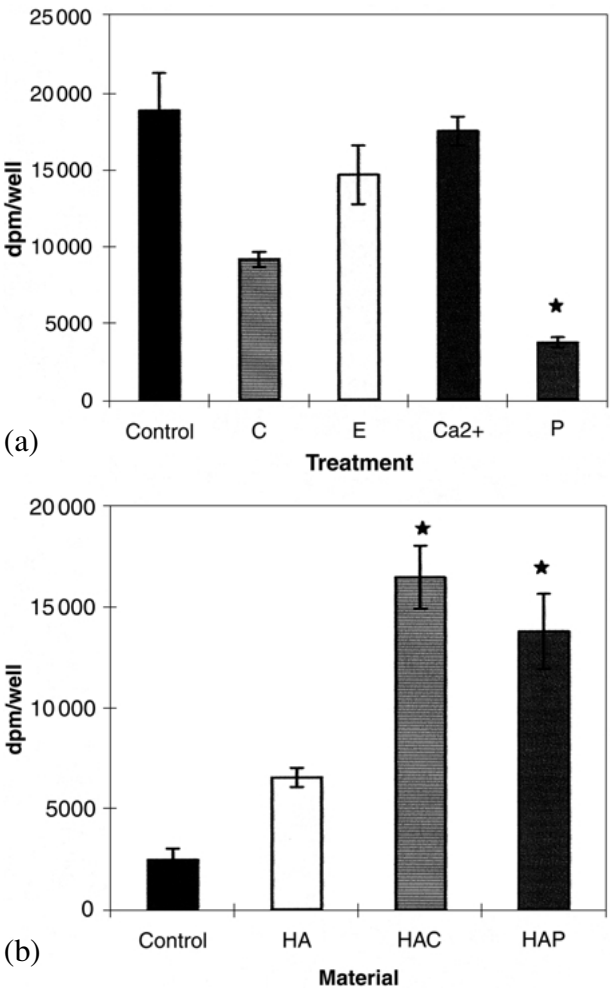

Figure 6 Collagen synthesis of FFC cells exposed to bisphosphonates. (a) shows the effect of clodronate (C), etidronate (E) and pamidronate (P) when added to the cell culture medium for 24 h. The drugs (1 mM) were added 24 h after seeding the cells. P was added in calcium-free medium, and controls were included with cells grown in this medium without P. Results are means ± SEM, of four experiments. $*P < 0.05$, compared with appropriate medium controls. (b) shows the collagen synthesis in cells growing on control polystyrene culture dishes, uncoated hydroxyapatite (HA), and HA coated with either clodronate (HAC) or pamidronate (HAP) between 24 and 48 h after seeding. Results are means ± SEM, of three experiments. $*P < 0.05$, compared with HA.

Before considering the approach of bisphosphonate-coated implants as a means of minimizing osteolysis *in vivo*, there are several questions to be answered and this manuscript addresses the first of these. Would bisphosphonates coated onto implants interfere with the viability and function of osteoblasts and therefore influence the process of osseointegration?

Although P in solution decreased the protein content of osteoblast cultures within 48 h, this effect was not apparent when the drug was coated onto HA. P in solution also potently inhibited protein and collagen synthesis measured using the incorporation of the radiolabeled precursors, leucine and proline, respectively. Furthermore, in the presence of P in the medium the osteoblasts do not form an extracellular network of collagen fibers. Gaziotis and coworkers [15] also demonstrated that P (50 μM), but not E, inhibited collagen synthesis when added to chick explant cultures for 6 days and this effect was independent of any inhibition of mineralization. These data suggested that, of the bisphosphonates being studied, P may not be suitable for coating prostheses, as it appeared to have a consistent effect on cell protein synthesis and inhibited the production of extracellular matrix protein. Thus, it would be likely to deter osseointegration of implants. However, these inhibitory effects of P were not observed when cells were cultured on HA coated with P. In fact, the syntheses of DNA, protein and collagen were significantly increased when cells were cultured on HA surfaces coated with either C or P. The effect on DNA synthesis was greatest (8-fold increase), but protein and collagen synthesis were also increased 3–4-fold and 2–3-fold, respectively.

These are the first indications that the pharmacological properties of the bisphosphonates may be altered when they are bound to HA. The inhibitory effects of P in solution on protein synthesis are not observed when the drug is bound to HA. On the contrary, when bound to HA both C and P appear to have a potent proliferative effect on osteoblasts *in vitro* increasing both DNA synthesis and protein synthesis. Collagen synthesis is also increased when HA is coated with either C or P. Stimulatory effects of low concentrations of the bisphosphonates have been noted previously on mineralization

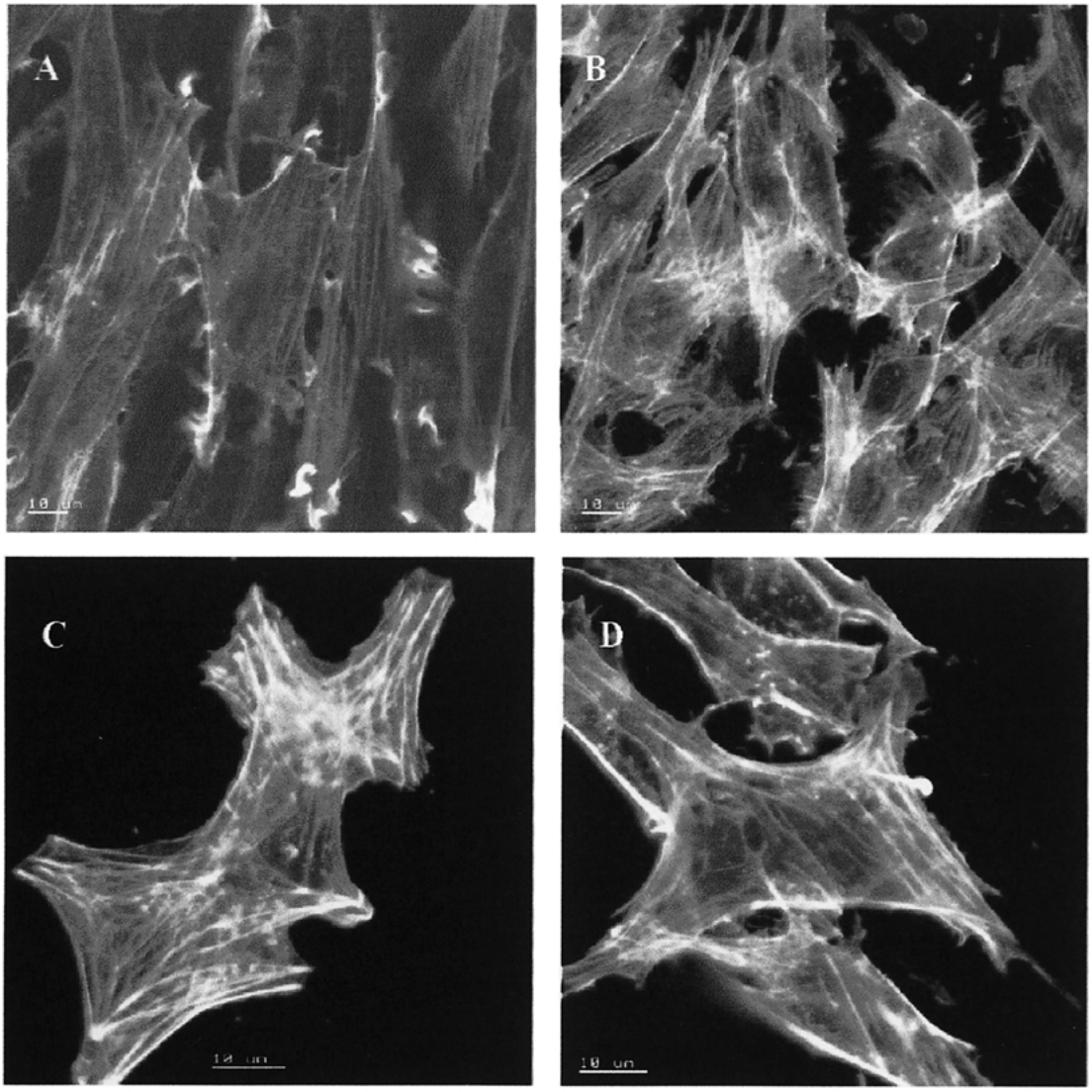

Figure 7 Confocal laser scanning microscopy photographs of FFC cells stained with FITC-labeled phalloidin to show the actin filaments. Scale bars are on each picture. (A) shows the control cells, (B) the cells after treatment with clodronate, (C) after etidronate and (D) pamidronate. All drugs were added at 1 mM, 24 h after cell seeding, and cells were exposed to them for 3 days.

in human osteoblasts [18]. Thus, the stimulatory effect of C and P, when bound to HA, could be due to the effect of low free-drug concentrations, and not of the bound drug. Coating HA prostheses with bisphosphonate drugs may in fact encourage the ingrowth of osteoblasts, and laying down of new extracellular matrix. These initial preliminary findings should stimulate interest in this approach to improving the life-time of orthopedic implants. Judging by the profound alterations in pharmacological effect observed in the present study after the drugs were bound to HA it is important to determine the effect of bound bisphosphonates on the functions of osteoclasts and macrophages. This is the major mechanism responsible for their effect on osteolysis *in vivo*, and it is essential that it should not be adversely affected by HA binding. At present, we do not know whether or not the drugs bound to HA are pharmacologically active towards the cells adhering to the surface of the HA material.

The drugs do not appear to be overtly toxic *in vitro* at the high concentrations tested on the osteoblasts. They do not alter redox balance inside the cells as the GSH levels were maintained. Only C showed a decrease in the MTT assay, and the reason for this effect is not clear at present. The actin cytoskeleton structure was markedly disturbed by exposure to E, and this effect appeared to be specific to E; neither P nor C influenced the distribution of actin filaments. Alterations in the actin cytoskeleton may influence the ability of the cells to adhere to surfaces, however, because of technical difficulties with the stain for actin binding to the HA material we are unable at present to determine the effect of bound bisphosphonate on the actin skeleton.

The data presented here show that when C and P are bound to HA they exert stimulatory effects on osteoblasts which may improve osseointegration of HA-coated implants impregnated with these bisphosphonates. We are currently determining the effect of HA coated with bisphosphonates on the functions of osteoblasts and macrophages to determine whether the drugs would remain effective inhibitors of osteolysis while bound to HA.

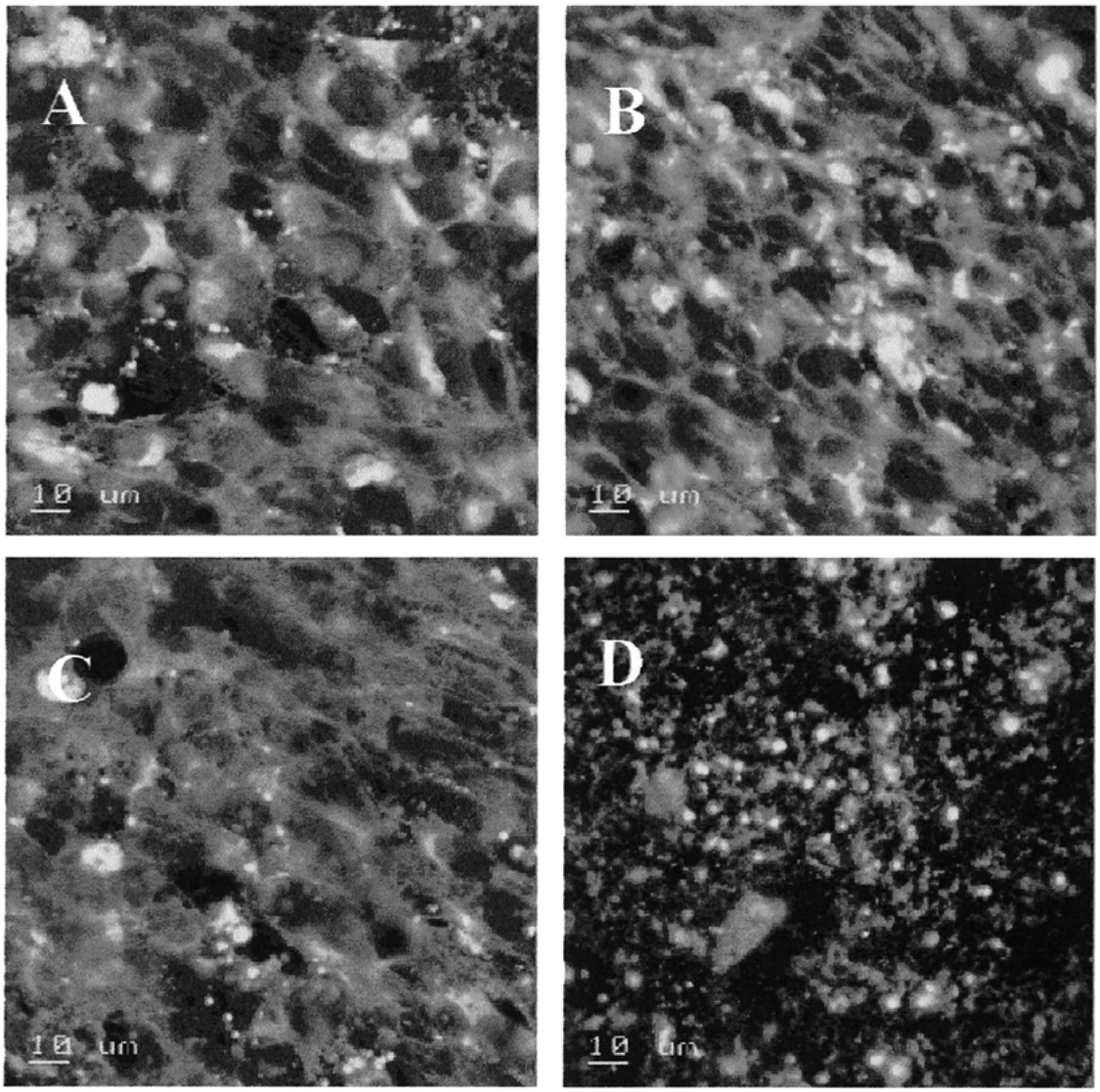

Figure 8 Confocal laser scanning microscopy pictures of cells stained with an antibody to collagen I labeled with FITC-avidin. Scale bars are on the pictures. (A) shows control cells, (B) the cells after treatment with clodronate, (C) after etidronate and (D) pamidronate. All drugs were added at 1 mM, 24 h after cell seeding, and cells exposed to them for 3 days.

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