In vitro growth and differentiation of osteoblast-like human bone marrow cells on glass reinforced hydroxyapatite plasma-sprayed coatings

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Human osteoblastic bone marrow cells were cultured for periods of up to 28 days in control conditions and on the surface of a glass reinforced hydroxyapatite composite (HA/G1) and commercial hydroxyapatite (HA) plasma-sprayed coatings, in the ''as-received'' condition and after immersion treatment in culture medium for 21 days. Cultures were characterized for total protein content and alkaline phosphatase activity. Scanning electron microscope analyses were performed on control cultures, seeded materials and materials incubated in the absence of cells. Culture media were analyzed for total and ionized calcium and phosphorus concentrations throughout the incubation period. Immersion of HA/G1 and HA coatings in culture medium resulted in significant alterations to the levels of calcium and phosphorus in the medium, leading to surface modifications. However, seeded material samples showed significant differences in the pattern of variation of the levels of these species. Cell proliferation was observed in the ''as-received'' HA/G1 composite, but cell mediated formation of mineral deposits was not proved. In contrast, ''as-received'' HA hardly supported cell growth. Previously immersed material samples showed cell proliferation and evidence of biological formation of mineral deposits. However, the HA/G1 composite presented better surface characteristics for cell growth as the behavior of bone marrow cells was closer to that observed in control cultures.

1. Introduction

Calcium phosphate ceramics are widely used as bone replacement materials [1, 2]. These materials have been described as bioactive because of their ability to bond directly with bone tissue [3, 4]. This process appears to be related to a modification of the materials surface, namely, the naturally induced formation of biologically equivalent carbonated apatite [5–7], as a result of dissolution, precipitation and ion exchange reactions with the physiological environment.

Hydroxyapatite (HA), a calcium phosphate ceramic, has been extensively used as a bone replacement material, but it is limited to low-load applications [8]. Natural apatite in bone and synthetic hydroxyapatite have related crystallographic parameters, but fundamental atomic composition differences exist between them [9]. The inorganic part of the bone does not entirely consist of apatite, but also contains β-tricalcium phosphate. In addition, although in bone tissue, the exact composition and relative proportions of collagen fibers, mineral part and ground substance depend upon the location and loading requirements, it is well accepted that the inorganic part contains several trace ions, particularly, Na^+, Mg^{2+}, K^+ and F^- [8–10]. Glasses within the $P_2O_5 - CaO - Na_2O$ system have been considered as having good potential as biomaterials [11–13], because their chemical composition presents similarities with that of the inorganic constituent of the mineral part of bone. Also, several attempts have been made in order to reinforce HA and to approximate its mechanical properties to those of bone [14]. Glass reinforced HA composites have been recently developed [15–17] by introduction of phosphate-based glasses in the microstructure of HA through a simple liquid phase process. These composites have higher fracture toughness than sintered HA, and a comparative study of the two materials concerning the formation of an apatite layer on their surface, after immersion in simulated physiological solution, suggested that the composites might show enhanced bioactivity, due to their higher rate of bone bonding [17].

Ceramic plasma-sprayed coatings on metal substrates (stainless steel or titanium alloy) have been accepted as implant materials in orthopaedic and dental applications because of their favorable biocompatibility and adequate mechanical properties [18–26]. Several *in vitro* studies

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suggest that HA plasma-sprayed coatings present better surface characteristics for bone cell growth and differentiation compared with dense HA or polished titanium [23, 25]. However, a recent study showed that human bone derived cells were difficult to grow on ''as-received'' HA plasma-sprayed coatings, and cell development was observed only on samples previously immersed in culture medium, showing that cultured cells are very sensitive to the biomaterial surface [26, 27].

The aim of this study was to analyze the morphological and functional behavior of osteoblast-like human bone marrow cells seeded onto a glass reinforced HA plasma-sprayed coating, and to compare the results with those obtained on a simple HA plasma-sprayed coating, under the same experimental conditions. Because previous studies have shown that storage conditions and pretreatments [26, 28, 29] may alter the *in vitro* cell behavior, the influence of an immersion treatment in culture medium of samples from both materials, prior to cell seeding, was also analyzed.

2. Materials and methods

2.1. Preparation of materials

A phosphate-based glass (G), with chemical composition listed in Table I was prepared from reagent grade chemicals. The composite preparation method has been previously described [15–17]. Glass addition of 2 wt % to HA (supplied by Plasma Biotal, UK) was used to obtain HA/G1 composite. Mixed powders were then dried, isostatically pressed at 200 MPa and sintered. Samples were milled and sieved to provide a particle size distribution suitable for plasma spraying.

Commercially available titanium alloy (Ti–6Al–4V) was used as substrate and discs 14 mm in diameter and 3 mm thick were prepared. Titanium discs were plasma-sprayed with commercial HA powder (coating thickness of 120 μm) and with the HA/G1 composite (double layer composed of 60 μm commercial HA, followed by a 60 μm HA/G1 composite).

2.2. Cell culture

Human bone marrow, obtained from surgery procedures on a 23-year-old man, was cultured in α-minimal essential medium (α-MEM) containing 10% foetal bovine serum, 50 $\mu g\,ml^{-1}$ gentamicin and 2.5 $\mu g\,ml^{-1}$ amphotericin B and supplemented with ascorbic acid (50 $\mu g\,ml^{-1}$), β-glycerophosphate (β-GP, 10 mmol) and dexamethasone (10 nmol). Incubation was carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Primary cultures were maintained until near confluence (10–15 days) and, at this stage, adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase) and seeded in 24-well dishes at a density of 2×10^4 cells cm^{-2}. Bone marrow cells were cultured for periods of up to 28 days, in the same experimental conditions as those used in primary cultures, on the surface of the plasma-sprayed samples: (i) HA/G1 composite and HA-coated discs in ''as-received'' conditions; (ii) HA/G1 composite and HA-coated discs previously immersed in complete culture medium for 21 days at 37 °C and in a humidified atmosphere of 95% air and 5% CO_2. Also, bone marrow cells were cultured in parallel on standard plastic tissue culture plates as control cultures. In addition, coated discs, both in the ''as-received'' condition and previously immersed in culture medium, were incubated in the absence of bone cells under the same experimental conditions. Culture medium was changed twice a week in all tested situations.

At the end of each culture period (one, three, seven, 14, 21 and 28 days), cell cultures, both control cultures and cultures growing on the surface of the coated discs, were characterized to evaluate total protein content and alkaline phosphatase activity (ALP), and were observed by scanning electron microscopy (SEM).

TABLE I Chemical composition of P_2O_5-based glass (mol %)

	P_2O_5	CaO	Na_2O	K_2O
G1	35.0	35.0	20.0	10.0

2.2.1. Biochemical assays

At the end of each culture period, cultures were washed twice with phosphate-buffered solution (PBS) and stored at − 20 °C until the end of the experiment. ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton) and assayed by the hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (p-nitrophenol) at $\lambda = 405$ nm (hydrolysis was carried out for 30 min at 37 °C). Results are expressed in nanomoles of p-nitrophenol produced per minute per square centimeter ($nmol\,min^{-1}\,cm^{-2}$); data are presented as the average of three replicates (mean ± standard deviation). Protein content was determined in 0.1 M NaOH cell lysates according to the method of Lowry using bovine serum albumin as a standard. Results are expressed as micrograms per square centimeter.

2.3. Scanning electron microscopy

For SEM observation, control cultures, materials with cultured cells and materials incubated in the absence of bone cells were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate (pH 7.3), then dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analyzed in a JeoL JSM 6301F scanning electron microscope equipped with X-ray energy dispersive spectroscopy (EDX) microanalysis capability, (Voyager XRMA System, Noran Instruments). SEM observation was also performed on the material samples in the ''as-received'' condition and after pretreatment with culture medium for 21 days.

2.4. Calcium and phosphorus measurements

Culture media from control cultures and also cultures growing on the surface of the material samples were collected twice a week, at each change of medium,

during the 28-day incubation period and analyzed for total and ionized calcium and phosphorus concentration. Quantification of these ions in the culture media collected from the material samples incubated in the absence of bone cells (both in the ''as-received'' condition and pretreated 21 days with culture medium) was also performed. Calcium was measured using a calcium kit (Sigma No. 587M) and phosphorus was measured using the inorganic phosphorus kit (Sigma No. 670-C); for total phosphorus quantification, samples of culture medium were first digested in a microwave oven (CEM Model MDS-2000) with nitric acid (suprapure 65%), in order to destroy organic matter, and the solutions obtained were treated as for inorganic phosphorus quantification.

3. Results

Human bone marrow cells were grown under experimental conditions described to favor the formation of osteoblast-rich cell cultures [30–36] and cultured for periods up to 28 days on the surface of an in-house glass reinforced HA composite (HA/G1) and commercial type HA plasma-sprayed coatings. The coatings were seeded both in the ''as-received'' condition and after immersion treatment with culture medium for 21 days. As a reference control, bone marrow cells were cultured in parallel on tissue culture plastic plates.

3.1. Total protein content and ALP activity

Results concerning total protein content and ALP activity in control cultures and cultures growing on the material coatings are presented in Fig. 1.

In control cultures, total protein content increased progressively with incubation time, although a tendency for a stationary state seems to have been attained at 28 days. ALP activity was relatively low in the first three days, then increased sharply to a peak level of response and dropped significantly during the third week of culture.

Cultures grown on the surface of previously immersed HA/G1 composite-coated discs presented identical behavior to that observed in control cultures; total protein content and ALP activity followed a similar pattern of variation, although values found for these two parameters were slightly lower (Fig. 1). In cultures grown in pretreated HA-coated discs, total protein content was relatively low in the first two weeks, but increased significantly by the third and fourth weeks and, at day 28, values were similar to those found in HA/G1 composite pretreated discs. ALP activity was low in the first three weeks but increased during the fourth week of culture.

Cultures grown on the material coatings used in the ''as-received'' condition, both HA/G1 composite and HA, presented identical behavior. Total protein content increased slowly during the culture period and values were much lower than those observed in control cultures and also in cultures growing in the pretreated materials (Fig. 1). In addition, ALP activity was very low throughout the culture time (Fig. 1).

3.2. Scanning electron microscopy

SEM observation of the control cultures at the various culture times showed that proliferation of bone marrow cells was accompanied by the production of a fibrillar extracellular matrix, and the 21-day cultures presented numerous globular mineral structures, shown by EDX to contain calcium and phosphorus (results not shown); this aspect is in accordance with other previously reported studies in this culture system [36].

Plasma-sprayed coated discs were observed by SEM in the ''as-received'' condition and after the 21-day immersion treatment in culture medium. Results are shown in Fig. 2. In both HA/G1 composite and HA, the plasma-sprayed surface was very rough with patches of smooth and shiny glassy film; after immersion in culture medium, small spherical particles were observed throughout the coating surfaces in both materials.

Observation of the pretreated HA/G1 composite and HA coatings seeded with bone marrow cells revealed that, following seeding, the cells attached, spread and proliferated (Fig. 3) in an apparent attempt to colonize the entire substrate surface; neighboring cells maintained physical contact with one another through multiple cytoplasmatic extensions and concurrent elaboration of intercellular matrix was observed and, by day 21, formation of numerous globular mineral structures

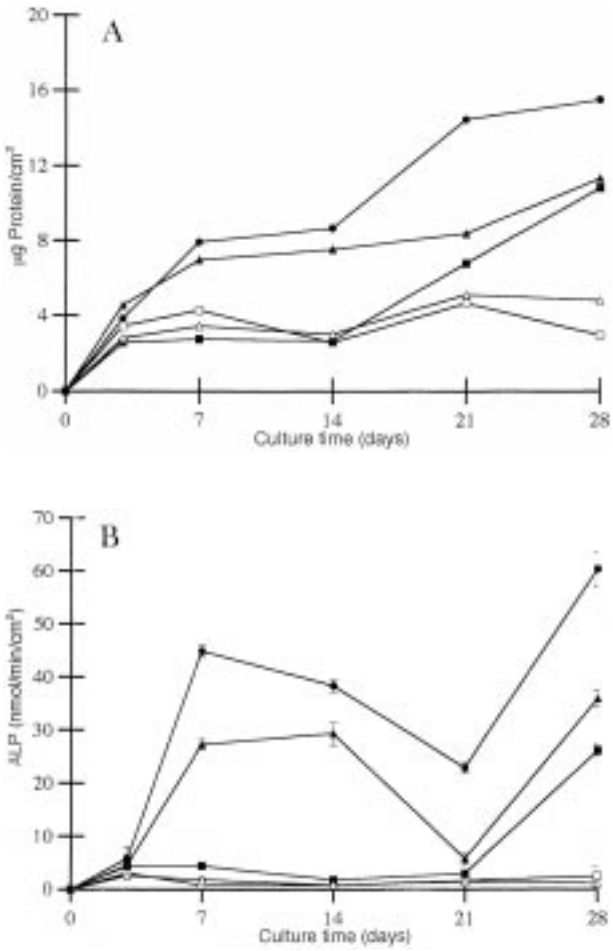
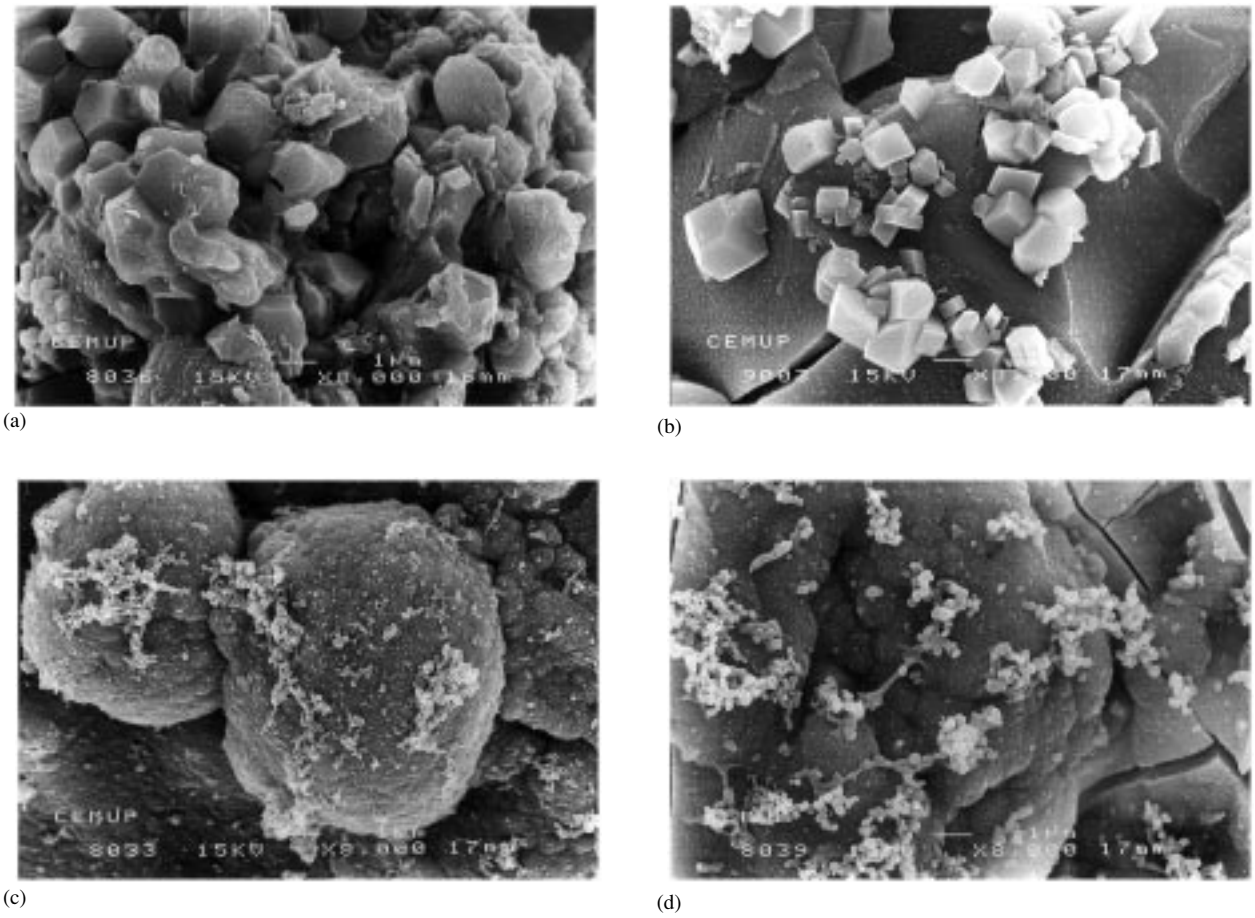
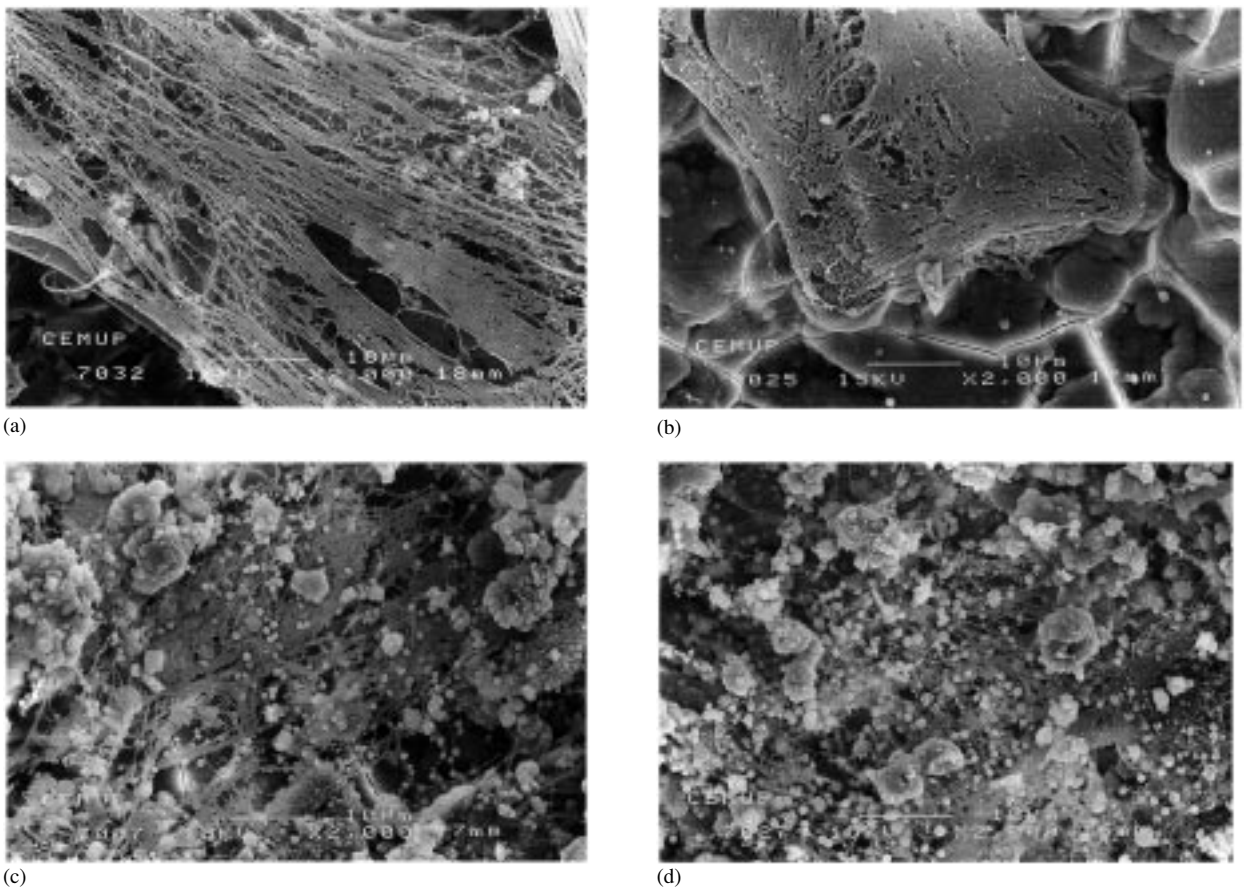


Figure 1 Total protein content (A) and ALP activity (B) of control cultures and cultures grown on the surface of treated (previously immersed in culture medium for 21 days) and non-treated plasma-sprayed coatings: (•) control cultures, (▲) cultures grown on treated HA/G1 composite, (■) treated HA, (△) ''as-received'' HA/G1 composite, and (□) ''as-received'' HA material samples.



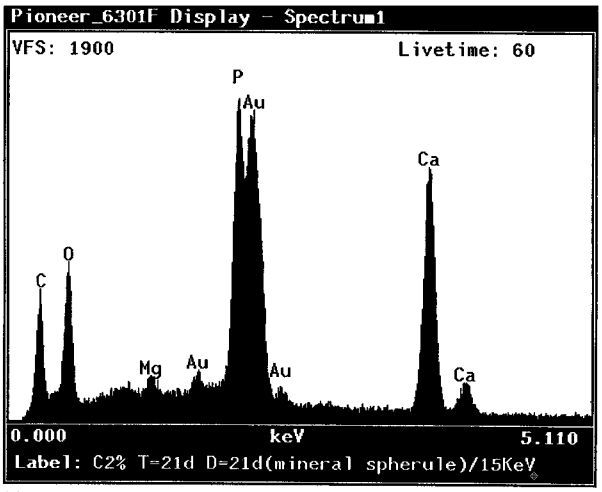
(a) (b) (c) (d)

Figure 2 SEM appearance of the plasma-sprayed HA/G1 composite and HA coatings in the ''as-received'' condition (a, b) and after being immersed for 21 days in culture medium (c, d): (a, c) HA/G1, and (b, d) HA.



(a) (b) (c) (d)

Figure 3 SEM appearance of seven- (a, b) and 21- (c, d) day cultures grown on the surface of previously immersed plasma-sprayed coatings. (e) EDX spectrum of the biological mineral structures present in 21-day cultures grown on HA/Gl composite: (a, c) HA/G1, and (b, d) HA.



(e)

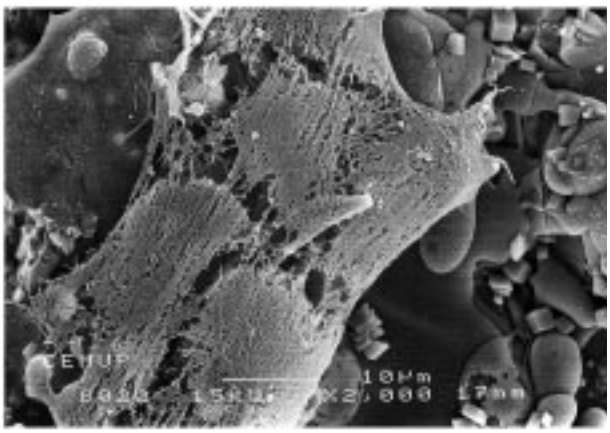
Figure 3 (Continued)

were visible throughout the culture. EDX of this biological structure confirmed the presence of calcium phosphates, carbon and oxygen compounds, characteristic of osteogenic tissue.

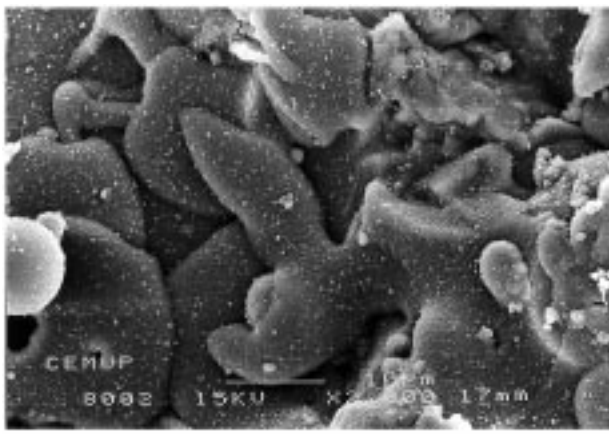
In contrast, and according to the results observed for total protein content and ALP activity, bone cells seeded on the surface of the non-treated plasma-sprayed coatings presented a less favourable behavior concerning cell proliferation and differentiation (Fig. 4). Bone marrow cells seeded on HA/G1 composite formed a cell layer (clearly visible in seven-day cultures), however, evidence of biological calcification was not observed. By contrast, in HA-coated discs, cell growth was observed only in some areas; most of the material surface was covered by biological material; apparently, fragments of dead cells and fibrous seric proteins adsorbed on the coating, especially in 21- and 28-day cultures.

3.3. Total and ionized calcium and phosphorus evaluation

Levels of total and ionized calcium and phosphorus in the original culture medium were, respectively, 1.94 and 1.61 mmol l^{-1} for calcium and 10.9 and 0.69 mmol l^{-1} for phosphorus. These species originated from calcium and phosphate compounds present in α-MEM and also in foetal bovine serum. The presence of β-GP (10 mmol) in the culture medium is responsible for the increased value observed for total phosphorus concentration as compared with that found for ionized phosphorus. Concentrations of total and ionized calcium and phosphorus in the culture media from cell cultures and also from materials incubated in the absence of bone cells were determined throughout the 28-day incubation period. It should be noted that the levels measured were not cumulative as the culture medium was totally replaced twice a week;



(a)



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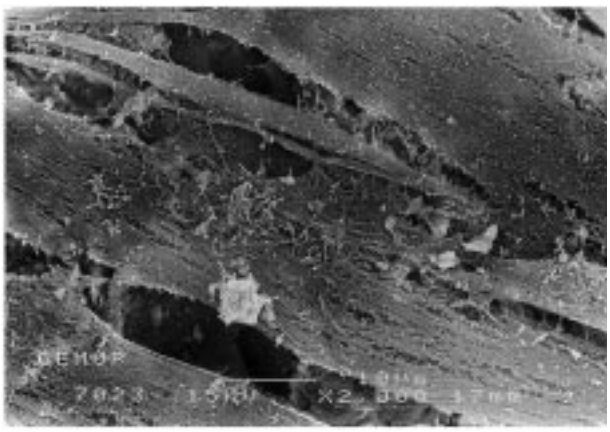
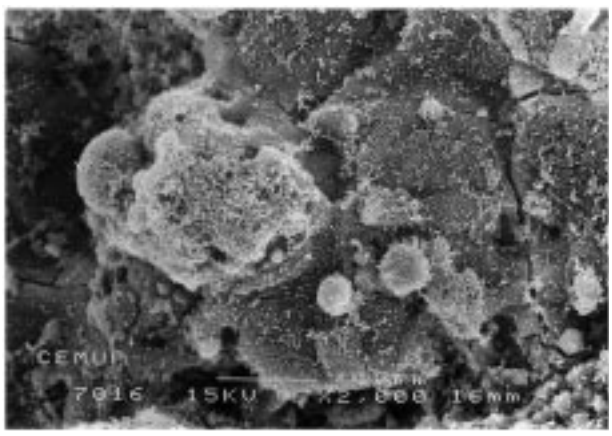


(c)



(d)

Figure 4 SEM appearance of seven- (a, b) and 2- (c, d) day cultures grown on the surface of plasma-sprayed coatings seeded in the ''as-received'' condition: (a, c) HA/G1, and (b, d) HA.

values determined reflect changes occurring in intervals of three–four days throughout the 28-day incubation period.

3.3.1. Materials incubated in the absence of cells

Fig. 5 shows the levels of total and ionized calcium and phosphorus in the culture medium collected from the samples incubated in the absence of bone marrow cells (for 28 days), both at the ''as-received'' condition and after being immersed for 21 days in culture medium. Immersion of the ''as-received'' HA-coated samples resulted in a significant increase in calcium and phosphorus concentrations in the medium in the first seven–ten days (peak levels were observed by day 7 for calcium and by day 10 for phosphorus); then, the concentrations of these species decreased; and, after approximately two weeks, values were similar to those measured in the culture medium before any contact with the samples. On the other hand, HA/G1 composite samples showed different behavior during the immersion period; increased levels of total and ionized calcium and phosphorus in the medium were observed at approximately three weeks, although release of these species was more significant in the first two weeks, and, from then onwards, levels were similar to those found in the original medium. As expected from the results described above, levels of total and ionized calcium and phosphorus in the culture medium from samples previously immersed for 21 days were similar to those found in the original medium.

3.3.2. Control cultures

Culture medium collected from control cultures (bone marrow cells growing on tissue culture dishes) showed little variation in the levels of total and ionized calcium during the first ten days of culture, and values were similar to those found in the original culture medium; however, during the same period, the concentration of inorganic phosphorus increased significantly, from 0.6 to $3\,\mathrm{mmol\,l^{-1}}$ at day 7, suggesting that part of the β-GP added was ionized, most probably by ALP, present in high levels in these cultures (Fig. 1), and known to have a high capacity to hydrolyze this esther phosphate [37]; but levels of total phosphorus remained constant during this period. However, after approximately two weeks of culture, a significant decrease in the levels of calcium and phosphorus was observed. These variations were caused by the pronounced reductions verified in the ionized contents of these species and, probably, reflect the mineralization process, i.e. the formation of calcium phosphate deposits that, according to previous work [36], occur in this culture system from two weeks onwards. After this stage, levels of these species remained approximately constant throughout the culture period, increasing again after day 25. Results are presented in Fig. 6.

3.3.3. Cultures grown on plasma-sprayed coatings

The pattern of variation of total and ionized calcium and phosphorus in the culture medium from cultures growing on pretreated HA/G1 and HA coatings was similar to that observed in control cultures (Fig. 6). However, during the first ten days, lower levels of total and ionized calcium were measured, especially in the case of HA-coated discs. Also, levels of ionized phosphorus were lower than those in control cultures, but higher than those observed in the absence of cells (because of the hydrolysis of β-GP by osteoblastic cells) and, accordingly, total phosphorus was also higher. After approximately ten days, a significant decrease on the levels of total and ionized calcium and phosphorus was observed, and values were significantly lower than those observed in the absence of bone cells, and, from then onwards, levels remained approximately constant until about day 25.

Levels of calcium and phosphorus in the medium from cultures growing on the non-treated discs were significantly different than those observed in the absence of cells. Also, some differences were evident as compared with what was found on the treated seeded discs (Fig. 6). On seeded HA discs, the concentrations of total and ionized calcium were, in the first ten days, similar to those found in the original medium, contrasting with the significant increase observed on non-seeded discs, and, after this stage, a significant decrease in the concentrations of these species was observed. Levels of ionized phosphorus increased in the first two weeks, although values were lower than those found on cultures grown on pretreated coatings, and remained constant for the rest of the culture time; levels of total phosphorus were similar to those on the original culture medium during the 28-day incubation period. On seeded HA/G1 composite discs, levels of total calcium decreased throughout the culture period until day 25, most probably due to the significant decrease observed on the levels of ionized calcium, especially during the first week: this behavior differs from that observed in treated seeded material samples where levels of calcium remained constant during the first two weeks of culture, decreasing afterwards. Levels of phosphorus (total and ionized) followed a similar pattern to that observed on the pretreated discs with an increase in the levels of ionized phosphorus in the first two weeks and a decrease of total and ionized phosphorus after this stage.

It is known from previous work that osteoblastic bone marrow cultures obtained in the experimental conditions described enter a senescence process after approximately three–four weeks [35, 36], and the increase in the levels of total and ionized calcium and phosphorus observed after 21–25 days may reflect this process, which most probably contributes to dissolution reactions of the mineral deposits on the sample surfaces.

4. Discussion

The aim of this work was to study the behavior of human osteoblastic bone marrow cells cultured on the surface of a glass reinforced HA plasma-sprayed coating (HA/G1) both in the ''as-received'' condition and after immersion

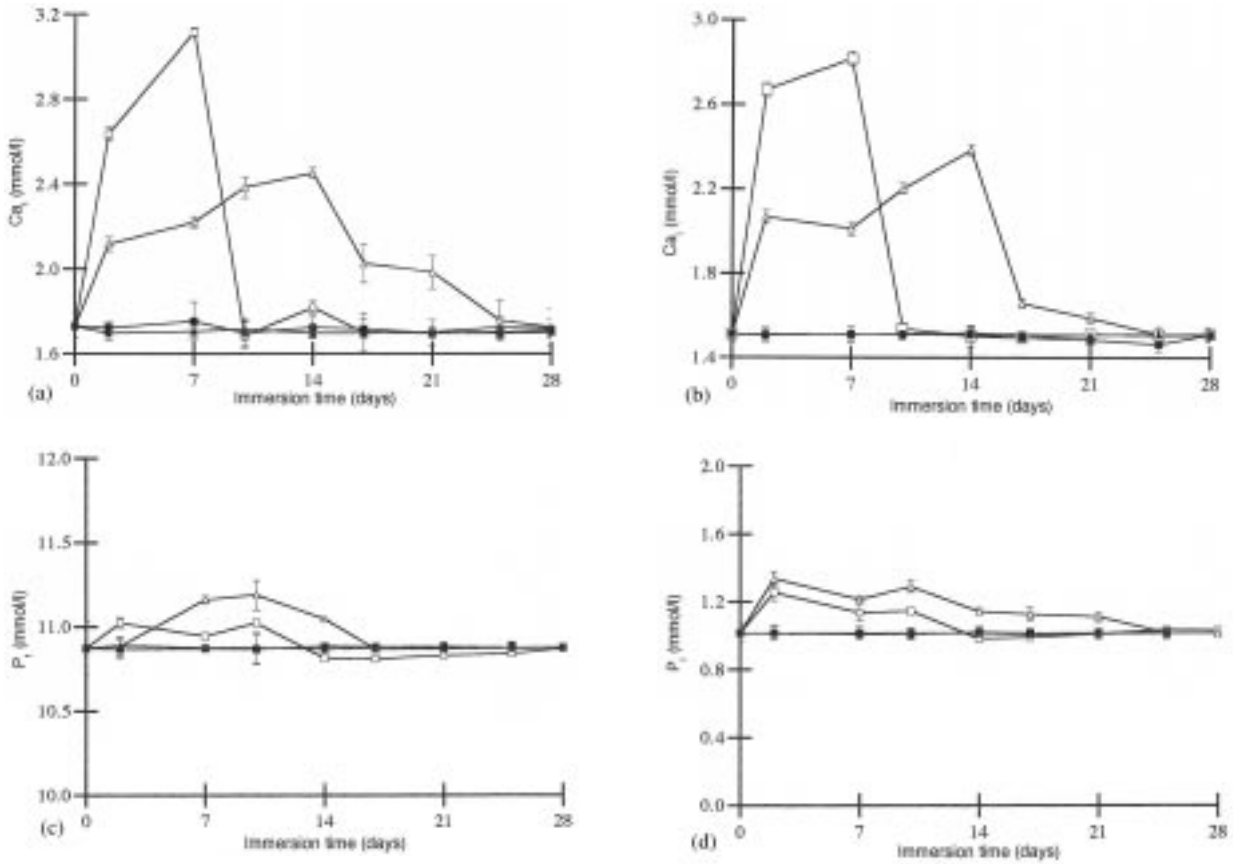


Figure 5 Levels of total and ionized calcium and phosphorus in the culture medium collected from the plasma-sprayed coated samples incubated in the absence of bone marrow cells for 28 days, both in the ''as-received'' condition and after being immersed for 21 days in culture medium: (△) ''as-received'' HA/G1 composite, (□) ''as-received'' HA, (▲) treated HA/Gl composite, and (■) treated HA material samples.

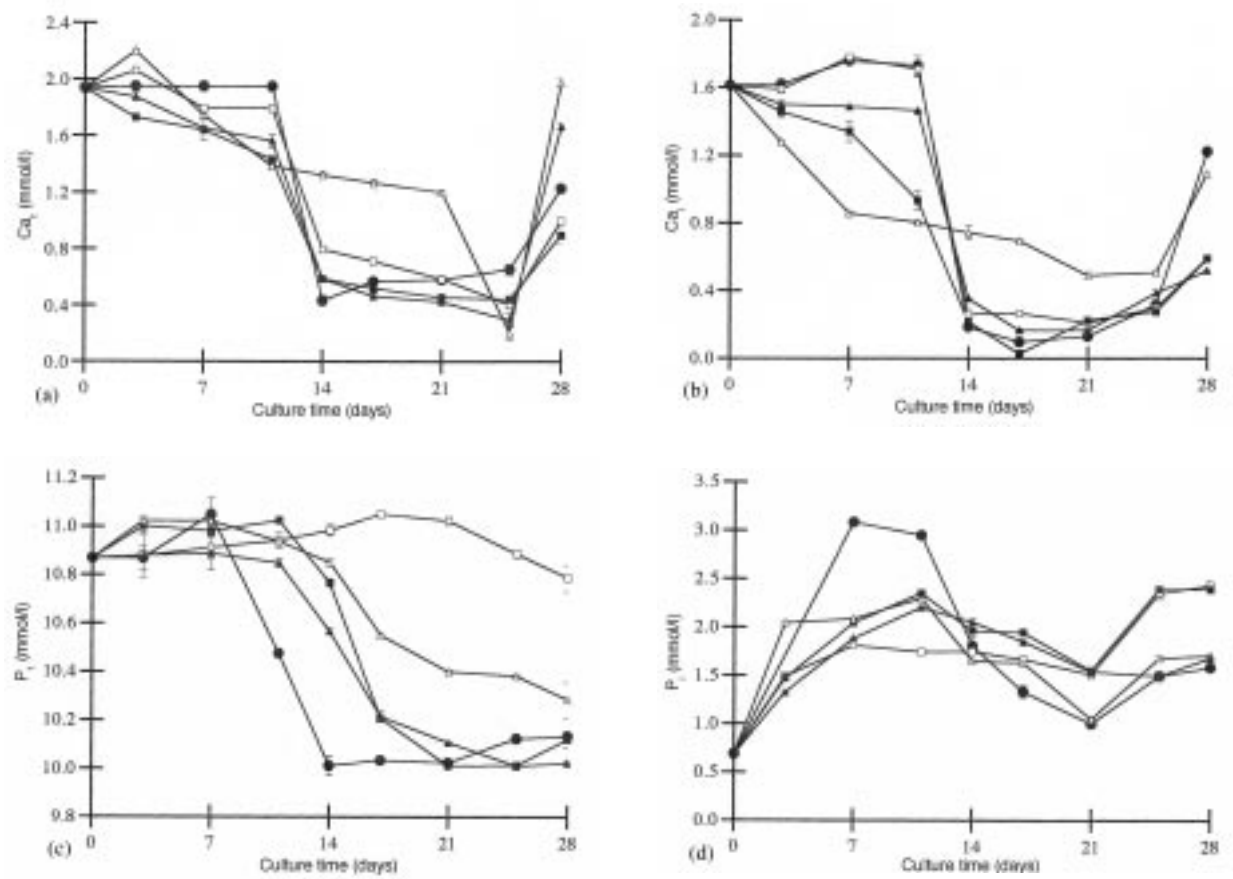


Figure 6 Levels of total and ionized calcium and phosphorus in the culture medium collected from control cultures and cultures grown on the surface of treated and untreated plasma-sprayed coatings, throughout the 28-day culture period: (•) control cultures, (△) ''as-received'' HA/G1 composite, (□) ''as-received'' HA, (▲) treated HA/G1 composite, and (■) treated HA material samples.

treatment in culture medium. The behavior of these cultures was compared with that observed in cultures grown on HA plasma-sprayed coatings under the same experimental conditions (non-treated and treated samples).

Immersion of ''as-received'' HA/Gl and HA-coated discs in culture medium for 28 days in the absence of bone cells resulted in significant alteration in the concentrations of total and ionized calcium and phosphorus in the medium (Fig. 5). Increased concentrations of these species were measured although with different characteristics for the two materials. For the HA coating there were high calcium and phosphorus concentrations in the medium during the first ten days, after which they decreased to levels similar to those found in the original medium. For the HA/Gl composite, calcium and phosphorus concentrations were smaller than for the HA coating and an equilibrium state seems to have been achieved after 21 days of immersion. These reactions between the plasma-sprayed coatings and the medium led to surface modifications, as shown by SEM observation of the samples before and after immersion (Fig. 2). Previous work showed that during immersion of these material samples in SBF, dissolution of the coating surface occurred and deposition of a calcium–phosphorus-rich layer on the surface of both materials had taken place [38]. Furthermore, a calcium–phosphorus-rich layer formed more quickly on the surface of composite coatings than on simple HA coatings [38], which may therefore explain the depletion in calcium and phosphorus detected in the culture medium in the present experiments.

Bone marrow cells growing in the plastic tissue culture dishes (control cultures), in the presence of ascorbic acid, β-GP and dexamethasone, presented osteoblastic characteristics, as demonstrated by the presence of high ALP activity (Fig. 1). These experimental conditions favor the growth of osteoblastic cells in several bone cell systems [30–36]. Bone marrow cells proliferated gradually with incubation time, this process being functionally related to the synthesis of a bone specific extracellular matrix, and, during the fourth week, a tendency for a stationary phase in cell growth was observed, most probably as a consequence of the accumulation and maturation of the extracellular matrix [39–41]. ALP also increased with culture time, and the significant increase in the levels of this enzyme during the second and third week suggested that cells were shifting to a more differentiated state [40, 41]. ALP appears to play a crucial role in the initiation of matrix mineralization; and, after that, expression of this enzyme is down-regulated [37, 42]. In this culture system, the mineralization process, an osteoblastic specific function, occurs during the third week of culture as shown by previous results indicating that 21-day cultures present a positive von Kossa reaction and numerous mineral globules on SEM observation [36], and also significant consumption of ionized calcium and phosphorus from the culture medium during this phase of the culture (Fig. 6).

From the results reported above, it is evident that previously immersed coated discs presented more appropriate surface characteristics for bone cell growth and differentiation. Cells growing in HA/Gl followed a similar pattern of behavior to that observed in control cultures, and higher values of total protein content and ALP activity were obtained, as compared with those observed on cultures growing on HA-coated discs. In this case, cell proliferation was lower in the first two weeks, and increased after that, and ALP activity attained significant levels during the fourth week. Considering that ALP has been regarded as an *in vitro* marker of osteoblastic differentiation [40], these results suggest that the cell population growing on HA/Gl appears to differentiate earlier and with a pattern similar to that observed in control cultures, as compared with the cell population growing on HA-coated discs. SEM observation of the seeded pretreated discs showed that bone marrow cells attached, proliferated and differentiated in both materials, as shown by the presence of abundant biological calcification on 21- and 28-day cultures (Fig. 3).

Levels of total and ionized calcium and phosphorus in the culture medium from seeded treated coated discs followed a pattern of variation similar to that observed in control cultures, although there was a closer similitude in the case of the seeded HA/Gl samples. In both situations, levels of ionized phosphorus increased in the first two weeks, suggesting hydrolysis of β-GP by ALP present in the cultures, and, similarly to what was observed in control cultures, after this stage, a significant decrease in the levels of calcium and phosphorus was noticed, suggesting cell mediated deposition of calcium phosphate salts. However, during the first ten days, levels of total and ionized calcium were lower than those measured in control cultures, especially in the case of seeded HA discs, indicating some calcium deposition, suggesting that the cells growing on the surface of the materials prevent, in some way, the dissolution reactions that occur in their absence (leading to increased calcium levels in the medium).

HA/Gl and HA coatings seeded in ''as-received'' conditions supported cell growth, although to a much lower extent than that observed in the samples previously immersed. Values for total protein content and ALP activity were similar for cultures growing on both materials. However, SEM observation showed significant differences concerning cell growth (Fig. 4). Bone marrow cells proliferated on the HA/Gl composite, although with a morphology that differed considerably from that observed on seeded pretreated discs, and formation of biological calcification could not be proved. In contrast, in HA-coated discs, cell growth was present only in a few limited areas and cells did not show typical osteoblastic morphology. Also, clear signs of cell deterioration and dead cells were observed. Concerning the levels of calcium and phosphorus in the culture medium (Fig. 6), results showed that the presence of bone cells on the coatings appears to influence immersion-induced reactions between the material and the medium. In HA/Gl discs ionized calcium decreased during the culture period, especially in the first week, suggesting calcium deposition from the medium and, after two weeks, some tendency for stabilization was observed, contrasting with the strongly decreasing values observed in the pretreated seeded discs during this phase of the culture. Also, during the same period, decrease in

the level of total phosphorus was lower than that observed on the pretreated samples. In HA-coated discs levels of calcium were similar to those observed in pretreated samples; however, levels of total phosphorus remained constant during the culture period. Only a small increase of ionized phosphorus was found during the first two weeks, which is in agreement with the low values found for total protein content and ALP activity and also SEM appearance of these cultures. The results observed suggest that the presence of cells contributed to prevent reactions between the surface coatings and the medium and, in addition, precipitation reactions appear to have occurred, specially from two weeks onwards, when concentrations of these species were lower than those found in the original culture medium, except for the level of phosphorus in the case of seeded HA samples. However, SEM observation of the cultures did not show evidence of a relationship between the decrease of calcium and phosphorus concentrations in the medium and the formation of cell-mediated mineral deposits.

These results are in agreement with those reported in the literature showing that appropriate surface characteristics are essential for *in vivo* and *in vitro* cell growth and differentiation [3–7]. Immersion-induced surface modifications of ceramic or glass–ceramic materials are well documented; molecular and ionic activity between the material surface and the aqueous environment results in the formation of a calcium–phosphorus-rich layer on the surface, which has been associated with adequate bioactivity of these materials [4–7]. Material surface–bone–cell interactions, namely the attachment of osteoblastic stem cells to the surface, is required for differentiation into osteogenic cells, resulting in firm bone bonding [43, 44].

The physicochemical modifications that seem to occur when HA/Gl and HA coatings are in contact with the culture medium, i.e. the release of significant amounts of some ionic species immediately after immersion, and/or local modification of pH, appear to be deleterious for cell attachment, growth and differentiation, as reported in other studies [26, 45, 46]. In the experimental conditions used, cell proliferation could be observed in ''as-received'' HA/Gl, but cell-mediated formation of mineral deposits could not be confirmed. In contrast, ''as-received'' HA hardly supported cell growth. After 21 days of immersion, equilibrium of exchange reactions (dissolution–precipitation) between coatings and medium appears to have been reached, and results show that the resulting surface modifications improved bone cell growth and differentiation significantly, probably due to the formation of a relatively stable calcium–phosphorus-rich layer on the surface coatings [38]. In addition, immersion of the material would promote adsorption of biological molecules from the culture medium, which have important roles in the adhesion process and functional behavior of osteoblastic cells [44, 47]. Cell proliferation and evidence of biological formation of mineral deposits were observed in cultures growing in previously immersed samples. The HA/Gl composite presented better surface characteristics for cell growth, as the behavior of bone marrow cells growing in this material was very close to that observed in control cultures, concerning cell proliferation and ALP activity; whereas, seeded HA samples showed delayed cell growth. The results reported are in agreement with a recent paper showing that cell growth of adult-bone-derived cells was not observed in HA plasma-sprayed samples seeded in the ''as-received'' condition, but could be obtained on samples previously immersed for 15 and 22 days in complete culture medium [26]. Another publication states that plasma-sprayed HA surfaces markedly prevent the proliferation of relatively undifferentiated pre-osteoblast precursors [25].

The differences in cell behavior of bone marrow cells growing on HA/Gl composite and HA coatings, both treated and untreated samples, are probably related to differences in surface characteristics, such as their chemical composition and physical properties, and also, to the pattern of immersion-induced reactions, which can be affected by these two parameters [26, 38, 48–50].

5. Conclusions

Plasma-sprayed glass reinforced HA coatings reported in this paper support the long-term proliferation of human osteoblastic cells. As in other similar materials, *in vitro* cell growth was very sensitive to surface characteristics, and immersion treatment of the samples, prior to cell culture, greatly improved cell growth and differentiation. Human bone marrow cells cultured on a previously immersed HA/Gl composite coating proliferate and secrete an extracellular matrix that is later mineralized without evidence of any impairment of cell physiology.

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