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Adhesion, growth and differentiation of human bone marrow stromal cells on non-porous calcium carbonate and plastic substrata: effects of dexamethasone and 1,25dihydroxyvitamin D3

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Bone marrow contains stromal fibroblastic stem cells which have the potential to differentiate into bone-forming cells. Therefore addition of bone marrow to porous bone substitutes such as coral or coralline hydroxyapatite may be expected to enhance bone ingrowth into these implants. This study was designed to evaluate the possibility of growing human bone marrow stromal fibroblastic cells (HBMC) on a calcium carbonate substrate. For this purpose, HBMC were cultured for 20 days on plastic or calcium carbonate and cell adhesion, growth, and differentiation were studied. It was concluded that calcium carbonate is a highly compatible material for the growth of HBMC. Cells were capable of adhesion within 30 min and were spread within 24 h on this material. However, plating efficiency was decreased in comparison to plastic. Population doubling times (PDT) showed that they were similar when the cells were grown on plastic or calcium carbonate as substratum (PDT = 4, 5.5 days). Early protein synthesis included collagen I, collagen III, osteopontin and bone sialoprotein. To induce differentiation of HBMC on plastic and calcium carbonate the influence of dexamethasone (Dex) and 1,25dihydroxyvitamin D3 (1,25$(OH)_2D_3$) on alkaline phosphatase (ALP) expression was studied. Basic ALP activity was similar when cells were grown on plastic or calcium carbonate. However, Dex and 1,25$(OH)_2D_3$ increased ALP activity of HBMC which could be driven best towards osteogenesis in the presence of Dex and 1,25$(OH)_2D_3$.

1. Introduction

Massive bone defects are a challenge to the most modern methods of reconstructive surgery. A number of studies have tried to solve this problem by using "hybrid materials" [1, 2]. A "hybrid material" is a biomaterial that is composed of a porous matrix in which bone cells may penetrate, proliferate and differentiate into bone tissue. The rationale for using bone marrow is that it contains stem cells which have the potential to differentiate along a variety of pathways including bone cells [3–6]. Therefore, addition of bone marrow cells to a porous matrix should enhance bone formation. Our ultimate goal is to seed fresh or *in vitro* expanded bone marrow cell populations on a porous matrix to obtain a bone hybrid material. Owing to its interconnected porous architecture, high compressive breaking stress, good biocompatibility and resorbability, coral is an interesting potential matrix to support bone marrow cell growth [7, 8]. Previous *in vitro* studies have demonstrated the use of coral as a lattice to support the growth of human fibroblasts [9], fetal rat bone cells [10] and gingival fibroblasts [11]. Clinically, coral is used with success in orthopaedic [12, 13], dental and maxillo-facial surgical applications [14].

This work aimed at evaluating the possibility of growing human bone marrow cells (HBMC) on dense calcium carbonate in conditions driving these cells towards osteogenesis. Dense calcium carbonate instead of natural coral was used to simplify analysis of the system. They are both composed of calcium carbonate in the form of aragonite. However, in contrast to coral, dense calcium carbonate is not porous. We therefore cultured HBMC on commercial tissue culture plastic and calcium carbonate for comparative purposes. We studied cell adhesion, growth and the effects of dexamethasone (Dex) and 1,25dihydroxyvitamin D3 (1,25$(OH)_2D_3$) on their osteogenic capacity. Alkaline phosphatase (ALP) activity was used as a marker for stromal cell differentiation. Calcium

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concentration was also monitored to study a possible influence of calcium carbonate dissolution on the calcium concentration of the culture medium.

2. Materials and methods

2.1. Preparation of calcium carbonate discs

The dense calcium carbonate used was supplied by INOTEB, Saint Gonnery, France. Discs of 13 mm diameter and 1 mm thick were sawn from blocks of fossils of the bivalve *Tridacna gidas* using a diamond saw (Himahashi, Japan). The mineral composition of the discs was 98–99% $CaCO_3$, 0.4–0.5% Na, 0.02–0.03% K and 0.1–0.2% Sr. The remaining 0.25 to 1.5% included amino acids and trace elements. After sterilization by autoclave, discs were preincubated in 1 ml of alpha-MEM medium overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 before addition of culture of marrow stromal cells.

2.2. Bone marrow stromal cell isolation and culture

HBMC were harvested from a 57-year-old patient. A single-cell suspension was prepared by repeatedly aspirating the cells successively through 19 gauge and 21 gauge needles. The cell suspension was filtered through sterile bolting cloth and cultured in alpha-MEM plus 15% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2. First-passage cells were used to inoculate calcium carbonate discs or 24-well cell culture dishes at a cell density of 1×10^4 cells per well. All experiments were carried out in the presence of 0.28 mM ascorbate.

2.3. Adhesion of HBMC to calcium carbonate

First-passage HBMC were seeded onto calcium carbonate discs. Cell cultures were fixed after 30 min, 3 h and 24 h with glutaraldehyde-cacodylate buffer, dehydrated through graded ethanol and critical point dried from CO_2 (Balzers Union CPD010). Samples were sputter-coated with gold and examined in a JSM 840 scanning electron microscope (SEM) (Jeol, Croissy sur Seine, France) at an accelerating voltage of 10 kv.

2.4. Calcium concentration of the culture medium

HBMC were cultured for 20 days. Cell culture medium was changed every 2 days and calcium concentration was measured before discarding the culture medium using plasma inductive spectrometry.

2.5. Immunocytochemistry

Rabbit IgG anti collagen types I, II and III were purchased from Institut Pasteur, Lyon, France. Antisera against type I collagen recognizes α1(I) and α2(I) chains after SDS PAGE and immunoblotting of pepsin-solubilized samples [15]. Antisera against type II collagen recognizes α1(II) chains after Western blotting of pepsin-solubilized samples [16]. Rabbit antisera to bone sialoprotein (LF6) and osteopontin (LF7) were a generous gift of Dr L. W. Fisher, NIH, Bethesda, Maryland, USA.

Immunostaining methods were carried out on HBMC culture after 2 days in order to detect the presence of osteopontin, bone sialoprotein, and collagen types I, II and III. Cells cultured on plastic or dense calcium carbonate were rinsed three times in phosphate-buffered saline, fixed with methanol at 4 °C for 15 min, permeabilized with 1% Triton × 100 for 5 min and with 0.1% Triton × 100 for 10 min. Cultures were then blocked with 2% goat serum for 30 min and incubated with the primary antibody overnight at 4 °C. The following morning, cultures were rinsed three times in phosphate-buffered saline and incubated with a goat antirabbit IgG conjugated with FITC antiserum (Sigma, Saint-Quentin-Fallavier, France) for 45 min. Samples were then thoroughly washed in phosphate-buffered saline and mounted in a solution of N-propyl gallate/Mowiol (Aldrich, Saint-Quentin-Fallavier France) and glycerol. Observations were made using a MRC 600 confocal microscope (Biorad, Hertfordshire, England).

2.6. Growth of HBMC on plastic or dense calcium carbonate

HBMC were grown on plastic or Tridacna discs for 20 days. Cells were counted after 5, 10, 15 and 20 days. Cells remaining suspended in the culture medium after 2 days were counted by using a ZM Coulter counter (Coultronix, Luton, England) in order to determine the number of non-adherent cells.

2.7. Effects of Dex and 1,25(OH)D3

2.7.1. Culture on plastic or calcium carbonate disc

HBMC were cultured for 20 days on plastic or calcium carbonate discs in alpha-MEM plus 15% fetal bovine serum under four different conditions: (i) no additions (control); (ii) addition of 10^{-8} M $1,25(OH)_2D_3$; (iii) addition of 10^{-8} M Dex: (iv) addition of 10^{-8} M Dex and 10^{-8} M $1,25(OH)_2D_3$. Addition of Dex or $1,25(OH)_2D_3$ was delayed until 24 h after plating. ALP activity and DNA content was determined at day 5, 10 and 20 on plastic and day 20 only on calcium carbonate substrata. Cell layers were scraped in 1 ml of distilled water, sonicated three times for 10 s and transferred to microfuge tubes. Cellular ALP activity and DNA content were determined as previously described [17, 18].

2.7.2. Influence of the concentration of FBS

HBMC were cultured on plastic or calcium carbonate in alpha MEM plus 15% fetal bovine serum and $1,25(OH)_2D_3$, or 10^{-8} M Dex from day 0 to day 18. FBS concentration was decreased to 2% from day 18 to day 20. $1,25(OH)_2D_3$ and Dex concentrations were kept unchanged. ALP activity was determined at day 20.

3. Results

3.1. Calcium concentration of the cell culture medium

In order to determine any possible physico-chemical modifications of the cell culture medium due to the presence of the calcium carbonate discs, calcium concentration was monitored during the 20 days of culture. Calcium concentration in the culture medium did not change significantly with time when cells were grown either on plastic or calcium carbonate. However, calcium concentration was significantly lower when cells were grown on calcium carbonate (55.4 ± 3.6 mg/l) in comparison to plastic (68.2 ± 1.6 mg/l) at day 20 (Fig. 1).

3.2. Adhesion of HBMC

Adhesion of HBMC on dense calcium carbonate was studied using scanning electron microscopy (Fig. 2). After 30 min, most of the cells present on calcium carbonate surface appeared as rounded cells extending short microspikes and lamellipodia less than 10 μm long (Fig. 2a). After 3 h, cells started to spread on the material surface emitting very long filopodia (>50 μm) (Fig. 2b). After 8 (Fig. 2c, 2d) or 24 h (Fig. 2e, 2f) of culture, most but not all cells appeared to be spread on the material surface. Extracellular matrix deposition was also observed (Fig. 2f).

3.3. Immunocytochemistry

HBMC were plated on dense calcium carbonate discs or plastic for 2 days. Immunostaining for collagen types I, II and III, osteopontin and bone sialoprotein was carried out. Expression of collagen type I, III as well as osteopontin and bone sialoprotein were observed when cells were grown on calcium carbonate (Fig. 3) or plastic (not shown). Collagen type II was not expressed by the cells on either substratum.

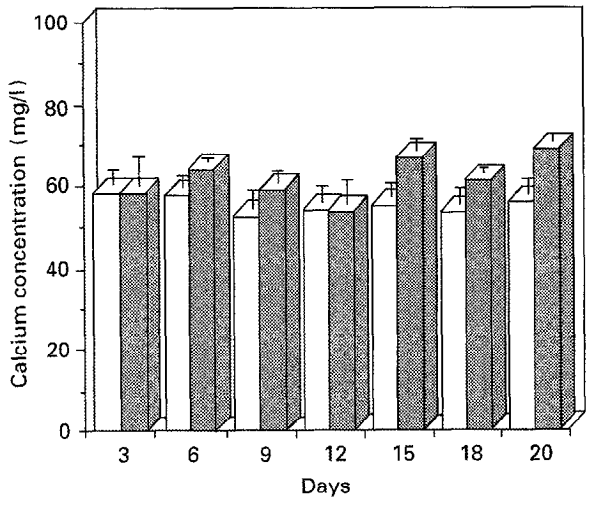

Figure 1 Calcium concentration of HBMC culture medium grown on plastic (▩) or calcium carbonate (□). Calcium concentration of the culture medium did not change significantly with time. However, calcium concentration was significantly lower when HBMC were grown on calcium carbonate after 20 days.

3.4. Growth of HBMC on plastic or dense calcium carbonate

HBMC were cultured for 20 days on Tridacna or plastic. Cell number was determined at day 5, 10, 15 and 20 (Fig. 4). Non-adherent cells were counted after 48 h in order to determine plating efficiency: 3085 ± 497 (calcium carbonate) and 2169 ± 547 (plastic) non-adherent cells were found in the culture medium after 48 h. Thus, plating efficiency was diminished on dense calcium carbonate. Therefore, cell growth on calcium carbonate was delayed in comparison to plastic. However, cell number was identical at day 20. The population doubling time between 50 000 and 100 000 cells were similar; 4.5 days on Tridacna and 5 days on plastic.

3.5. Effects of Dex and $1,25(OH)_2D_3$

3.5.1. HBMC culture on plastic

HBMC were cultured for 20 days in the presence of Dex, $1,25(OH)_2D_3$ or Dex + $1,25(OH)_2D_3$. ALP activity was determined at day 5, 10 and 20 (Fig. 5). ALP activity was stimulated in the presence of $1,25(OH)_2D_3$, or Dex. However, Dex + $1,25(OH)_2D_3$ was the most potent combination. ALP activity increased from 1.1 ± 0.2 nM/min/μg for the control to 42 ± 3.9 nM/min/μg for cells cultured in the presence of $1,25(OH)_2D_3$ and Dex. This difference was significant ($p < 0.005$).

3.5.2. Culture of HBMC on calcium carbonate

To investigate the effects of Dex and $1,25(OH)_2D_3$ on HBMC cultured on dense calcium carbonate, cells were grown for 20 days on dense calcium carbonate. ALP activity was determined at day 20 (Fig. 6). Combination of Dex + $1,25(OH)_2D_3$ in the culture medium led to an increase in ALP activity in comparison to control culture (0.4 ± 0.05 nM/min/μg versus 12 ± 3.4 nM/min/μg). However, this ALP activity was significantly less than the ALP activity of HBMC cultured on plastic (12 ± 3.4 nM/min/μg versus 42 ± 3.9 nM/min/μg). Dex alone did not increase ALP activity when HBMC were cultured on calcium carbonate.

3.5.3. Culture of HBMC on plastic and calcium carbonate in the presence of 2% fetal calf serum

In order to investigate the effects of reducing the calf serum concentration in the culture medium for the final part of the culture period, we also grew HBMC from day 1 to 18 in the presence of alpha MEM + 15% fetal calf serum. Serum concentration was decreased to 2% for the last two days. Concentration of Dex and $1,25(OH)_2D_3$ were kept unchanged. In this condition, cells grown in the presence of Dex + $1,25(OH)_2D_3$ on dense calcium carbonate increased the ALP activity from 12 ± 3.4 nM/min/μg to 69 ± 13 nM/min/μg. This ALP activity was not significantly different from ALP activity observed on plastic (76 ± 13 nM/min/μg).

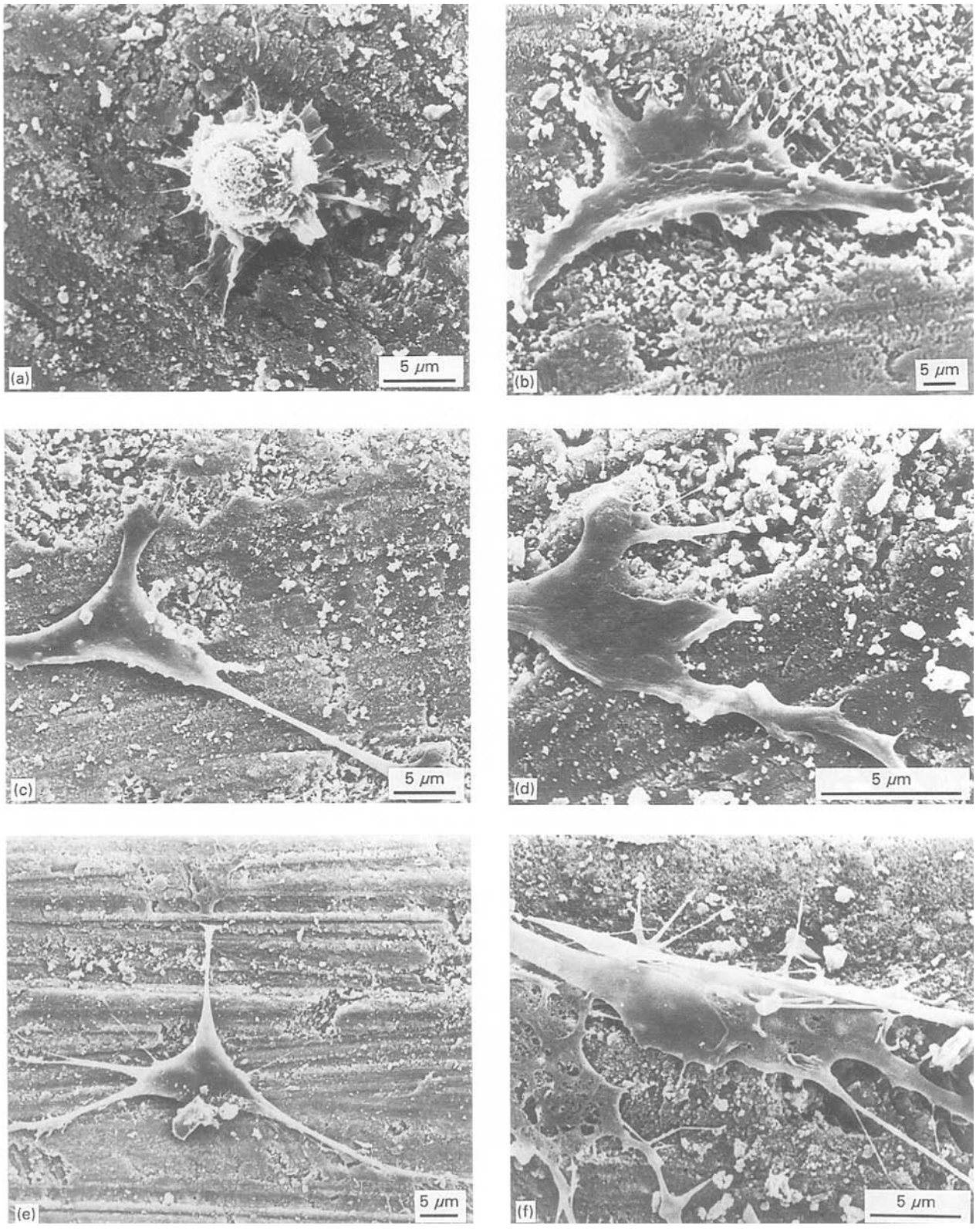

Figure 2 Scanning electron micrographs showing HBMC attachment to calcium carbonate: (a) 30 min; (b) 3 h; (c and d) 8 h; (e and f) 24 h.

4. Discussion

In this study the calcium concentration of the culture medium was significantly lower when cells were grown on calcium carbonate in comparison to tissue culture plastic. Our results suggest that calcium carbonate may act as a nucleating agent under these conditions as previously proposed in *in vitro* [19] or *in vivo* [20] studies. Similarly, immersion of calcium phosphate ceramic in a buffered solution with the electrolyte constituents of interstitial fluid led to a reduction of calcium concentration in solution possibly reflecting the precipitation of calcium on the calcium phosphate ceramic [21].

Immunostaining of HBMC grown on calcium carbonate showed that the cells showed positive expression of osteopontin, bone sialoprotein, collagen I and collagen III. These results are similar to those observed when rat bone marrow cells were grown on

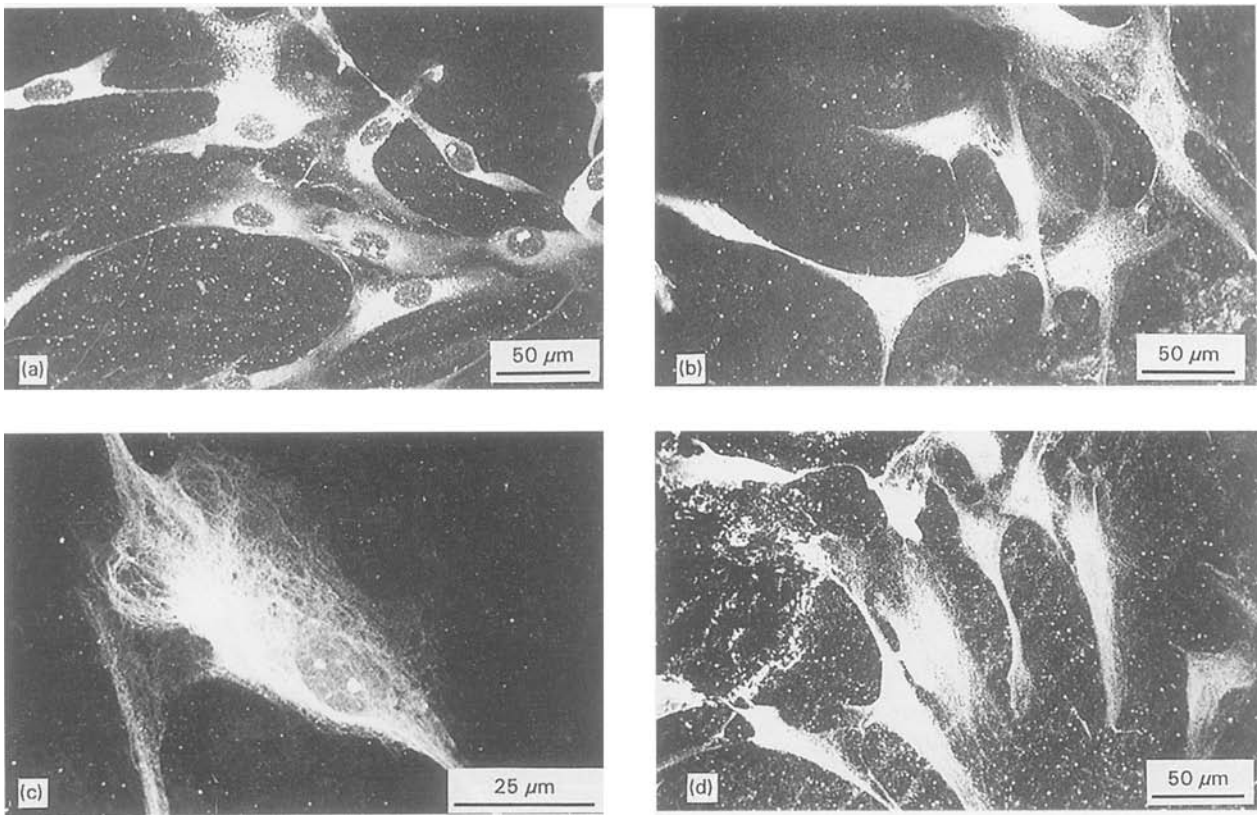

Figure 3 Immunolocalization of type I (a) and type III (b) collagen, osteopontin (c) and bone sialoprotein (d) in HBMC after 2 days of culture on calcium carbonate.

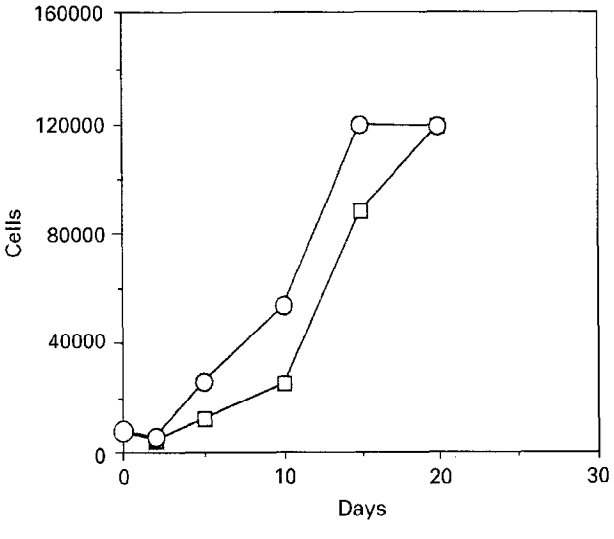

Figure 4 Kinetics of HBMC growth on plastic (–O–) and calcium carbonate (–□–).

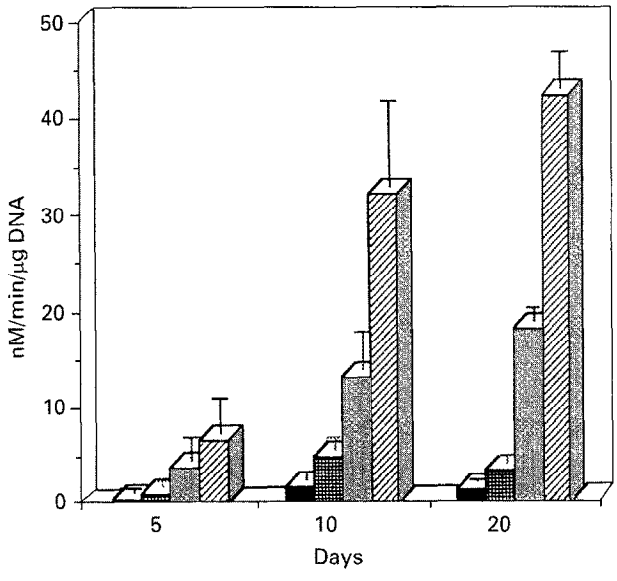

Figure 5 Effect of Dex and $1,25(OH)_2D_3$ on cellular ALP activity of HBMC after 5, 10 and 20 days of culture on plastic: ■ control; ▦ $1,25(OH)_2D_3$; ▩ Dex; ▨ Dex + $1,25(OH)_2D_3$.

polystyrene Petri dishes [22]. Further longer-term studies are needed in order to determine the localization of these components in the interfacial matrix or extracellular matrix. Effects of calcium carbonate on cell adhesion and growth were observed. Cell growth appeared to be delayed on calcium carbonate in comparison to plastic because of the reduced initial plating efficiency. Electron microscopy studies indicate that the Tridacna surface is rougher than plastic (not shown). A more appropriately prepared surface or coating by biomolecules may be expected to improve adhesion. Population doubling times were similar during the exponential phase of growth suggesting similar cellular growth rates.

Calcium carbonate in the form of aragonite is the major component of coral skeleton. When implanted, coral skeleton appears to be an osteoconductive material. It is progressively invaded by cells, resorbed and replaced by newly formed bone. The cellular mechanism underlying this *in vivo* behaviour is still poorly

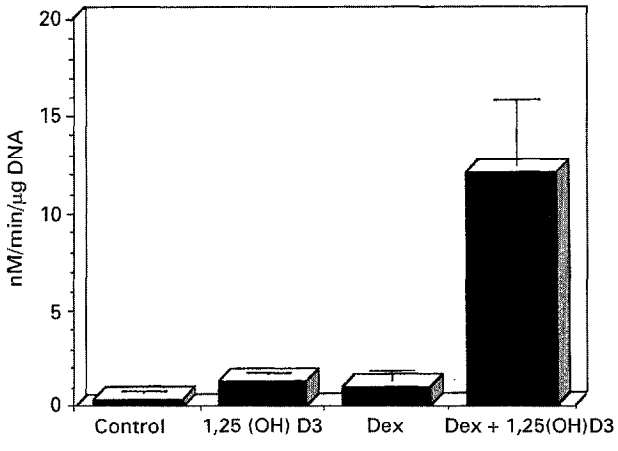

Figure 6 Effect of Dex and $1,25(OH)_2D_3$ on cellular ALP activity of HBMC cultured on calcium carbonate for 20 days.

understood but it has been suggested that the resorption of calcium carbonate is partially mediated by osteoclasts [7, 23]. The role of other cell lines is unknown. In this study, we observed that cultures of HBMC on plastic or calcium carbonate did not lead to any induction of ALP activity in the absence of Dex or $1,25(OH)_2D_3$. This result shows that *in vitro* calcium carbonate does not affect ALP induction.

Dex is a synthetic glucocorticoid which possess an enhanced biological activity in comparison to cortisol. Glucocorticoids regulate gene expression and the glucocorticoid receptor shows enhanced affinity for DNA upon binding of glucocorticoid [24]. Our finding that on plastic substratum the presence of Dex enhanced ALP activity is consistent with reports that showed that Dex is necessary for expression of osteogenic markers by rat bone marrow cells [17, 25]. Dex alone did not increase cellular ALP activity of HBMC grown on $CaCO_3$ but required $1,25(OH)_2D_3$. Recently, Gundle *et al.* [26] showed that addition of Dex promotes osteogenicity in cultured HBMC as assessed in the diffusion chamber assay.

$1,25(OH)_2D_3$ was also tested for its ability to regulate ALP activity. Our study indicates that ALP activity of HBMC grown on both plastic and calcium carbonate is increased in the presence of $1,25(OH)_2D_3$. This result is in accordance with findings by Franceschi *et al.* who demonstrated that addition of $1,25(OH)_2D_3$ is accompanied by an increase in ALP activity in bone-derived cells [27].

In our study, HBMC grown in alpha-MEM + 15% FBS in the presence of Dex and $1,25(OH)_2D_3$ on calcium carbonate exhibited a lower ALP activity in comparison to HBMC grown on plastic under similar conditions. A decrease in fetal calf serum concentration for the last 2 days of culture was paralleled by a six-fold increase in ALP activity. In possible explanation of these findings, it has been shown that inhibition of proliferation of osteoblast like cells results in increased ALP mRNA levels [28] and increased ALP enzyme activity [29]. Therefore, the effect of the decreased fetal calf serum used in our experiment on increasing ALP expression of HBMC may indicate that cell proliferation on calcium carbonate was still occurring at the 20-day time point. Our finding that cell number on plastic had reached a maximum at 15 days, but was still increasing at a linear rate on calcium carbonate at 20 days is in agreement with this possibility.

5. Conclusion

In conclusion, calcium carbonate is a suitable substratum for the growth and differentiation of HBMC. Cells are able to adhere and multiply on this substrate and are driven effectively towards osteogenesis in the presence of Dex and $1,25(OH)_2D_3$. Future objectives of this work include *in vivo* experimentation to test the capacity of cultured HBMC to enhance osteogenesis of porous implants of calcium carbonate.

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