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Effect of hydroxyapatite porosity on growth and differentiation of human osteoblast-like cells

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To study whether hydroxyapatite (HA) porosity can influence its osteoconductive properties, cell adhesion, proliferation and differentiation were compared in human osteoblast-like cells grown on HA disks of different porosity (A = 20%, B = 40%, C = 60%). Human osteoblast-like cells were isolated and characterized. Proliferation rate and alkaline phosphatase (ALP) activity were assessed at 3, 7, 15, 21, and 28 days. Type I collagen and osteonectin production were demonstrated with fluorescence microscopy and osteoblast adhesion studied at 7 and 21 days by scanning electron microscopic analysis. Cell growth on HA was three- to six-fold lower than on polystyrene control disks. At 28 days, 2141 ($\pm$350) cells/well grew on the most porous disks (Group C), with highly significant differences from controls ($p < 0.005$). The ALP production was 2–3 fold lower on HA than on plastic. In the Group C the mean ALP activity was of 2.95 ($\pm$0.07) UI/well after 28 days, higher than in the other two groups. At 21 and 28 days, proliferation rate and ALP activity on the three HA cultures were significantly different ($p < 0.05$). A decrease in cell population and increased ALP activity were observed on the most porous material, and high proliferation and poor differentiation rates on the less porous disks.

1. Introduction

Synthetic hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$] is the most used material in orthopedics because of its resemblance to biological HA, the main mineral component of bone [1]. To date, however, attempts to reproduce biological HA have met with only partial success, and autogenous cancellous bone (AB) is still the most effective material in stimulating osteogenic response [2]. The use of AB is however associated with morbidity, including pain, blood loss, increased use of blood products, surgical scars and higher operative time [3]. Furthermore, autograft harvest may provide insufficient material [4]. By contrast, allograft bone—which is available in larger amount—does not have the osteogenic potential of autogenous bone [5, 6] and may involve complications such as transmission of disease and an immunological response that may cause complete resorption without concomitant bone deposition [4].

Calcium phosphate ceramics, especially HA and the more resorbable β-tricalcium phosphate (β-TCP), are used as cancellous bone graft substitute materials [7, 8] to induce arthrodesis [2], fill bone defects [9], and as prosthetic coating in cementless joint replacements [10, 11]. Porous HA is bioactive because when implanted it directly binds to bone through chemical bonds [12, 13] without any intervening soft tissue layer [14]; in addition, it is biocompatible [15–17], non-carcinogenic [18] and exhibits excellent osteoconductivity, allowing ingrowth of bone cells, capillary sprouting, and growth of perivascular tissue from the host bone into the three-dimensional structure of the implant [19], ultimately replacing it with new bone to form a functional skeletal element [20]. However, porous HA seems to lack osteoinductive properties, i.e. the ability to support the proliferation of undifferentiated perivascular mesenchymal cells and their differentiation into osteoprogenitor cells able to form new bone [19].

Although a variety of calcium phosphate materials are available, the search for new types that more closely approximate bone mineral is a major research objective.

To study whether substrate porosity can influence its osteoconductive properties, we compared the adhesion, proliferation and differentiation of human osteoblast-like cells grown on HA disks with different porosity or on polystyrene as a positive control culture.

2. Materials and methods

2.1. Hydroxyapatite samples

HA disks (diameter 13 mm, height 3 mm) were obtained by cutting cellulosic spongeous cylinders previously rehydrated and dried. Three batches of samples with different average porosity (20, 40, or 60%) were produced by imbibition of the spongeous pellets. Crystallinity was between 85–90%. After imbibition, samples were naturally dried and sintered at 1250°C for 2 h under oxygen flow. Disk dimensions were then measured to check for shrinkage and the degree of porosity was evaluated by weight/volume ratio.

Disks were divided by degree of porosity into group A (20%), B (40%), and C (60%). Prior to use, disks were processed by γ ray sterilization.

2.2. Isolation and characterization of osteoblast-like cells

Bioptic specimens of human trabecular bone were obtained during surgical procedures from 4 young patients (mean age 24.5 years), and treated as previously reported [21].

For histochemistry and immunohistochemistry primary cells were replated in 4-well-chamberslides in DMEM-10% FCS supplemented with 5 mM β-glycerophosphate (Sigma, Italy) and 50 μg/ml ascorbic acid until confluence was reached. Slides were fixed with 2% phosphate-buffered formalin with 0.5% glutaraldehyde for 10 min at room temperature (RT). For detection of calcium deposition cells were stained with a modified Von Kossa procedure (Bioptica, Italy).

Production of alkaline phosphatase (ALP) was determined by exposing fixed cultures to a solution containing BCIP-NBT (Sigma) for 30 min.

Fixed cells were processed by the standard avidin-biotin peroxidase complex procedure (Vectastain'Elite kit, Vector, CA, USA). Non-specific binding was blocked with 3% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 30 min at RT, then cells were incubated with the primary antibodies overnight at 4°C. The following antibodies were used : polyclonal anti-type I collagen (dilution 1 : 100; Monosan, The Netherlands), polyclonal anti-type III collagen (dilution 1 : 20), monoclonal anti-osteonectin (dilution 1 : 100; both from DBA, Italy), and polyclonal anti-fibronectin (dilution 1 : 600; Sigma). Rabbit and mouse immunoglobulins at the same dilutions as the primary antibodies were used as controls. Peroxidase activity was revealed by incubation with 0.05% 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma) in PBS containing 0.03% peroxide for 5 min at RT. Slides were then washed, dehydrated and mounted with Eukitt.

To evaluate cell growth and ALP production, 5000 cells were seeded on the three types of HA disks and on polystyrene substrates.

2.3. Osteoblasts on HA samples

2.3.1. Cell growth

Cell proliferation on HA and control samples was determined at 3, 7, 15, 21, and 28 days by measuring the number of viable cells with the MTT colorimetric assay (Sigma) and measuring the optical adsorbance at 570 nm. Experiments have been performed in quadruplicate.

2.3.2. ALP production

Production of alkaline phosphatase on HA and control substrates was measured on cell lysates with kit 104 (Sigma) according to the manufacturer's recommendations. Quadruplicate determinations were performed at 3, 7, 15, 21, and 28 days. Values were calculated from optical adsorbance at 420 nm and expressed as international units of enzyme activity per ml (IU/ml). The mean ALP production per cell (IU/cell) was obtained dividing the ALP production with the cell number at each time point.

2.3.3. Statistical analysis

The differences in proliferation rate and ALP activity among the three groups of HA disks were evaluated by one-way analysis of variance (ANOVA). The level of statistical significance was established at $p < 0.05$.

2.3.4. Immunofluorescence

Osteoblasts spreading on HA were evaluated at 21 days by fluorescence microscopic examination according to the following procedures. Non-specific binding was blocked with 3% NGS in PBS for 30 min at RT; cells were then incubated with: polyclonal anti-type I collagen (dilution 1 : 100; Monosan) and monoclonal anti-osteonectin (dilution 1 : 100; DBA) overnight at 4°C. Rabbit and mouse immunoglobulins at the same dilutions as the primary antibodies were used as controls. A fluorescein isothiocyanate-conjugated goat anti-mouse and rabbit diluted 1 : 100 (Nordic Immunol. Lab. BV, The Netherlands) was used to localize the first antibody.

Fluorescence-labeled specimens were examined using a Zeiss Axiophot (Germany) fluorescence microscope.

2.3.5. Scanning electron microscopy (SEM)

After 7 and 21 days in culture, adherent cells were fixed with Karnovsky buffer solution (It is formed by 66 ml of a stock solution and 2.5 ml of 50% glutaraldehyde. The stock solution was made whit distilled water, paraformaldehyde and cacodylate buffer solution. The pH was adjusted to 7.2) for 2 h, washed briefly in cacodylate buffer (this buffer consists of sodium cacodylate, sucrose, $CaCl_2$. Distilled water was added to bring volume to 167 ml and the pH was adjusted to 7.2), postfixed in osmium tetroxide for 2 h, briefly washed again in cacodylate buffer, dehydrated in ascending series of alcohols, critical-point dried and coated with carbon. Samples were observed with a Cambridge 180 scanning microscope (MA, USA).

3. Results

3.1. Characterization of human osteoblast-like cells

Osteoblast-like cells positive for ALP, a typical membrane glycoprotein, showed dark purple areas and granules which indicated the expression of the enzyme (Fig. 1). They produced abundant mineralized

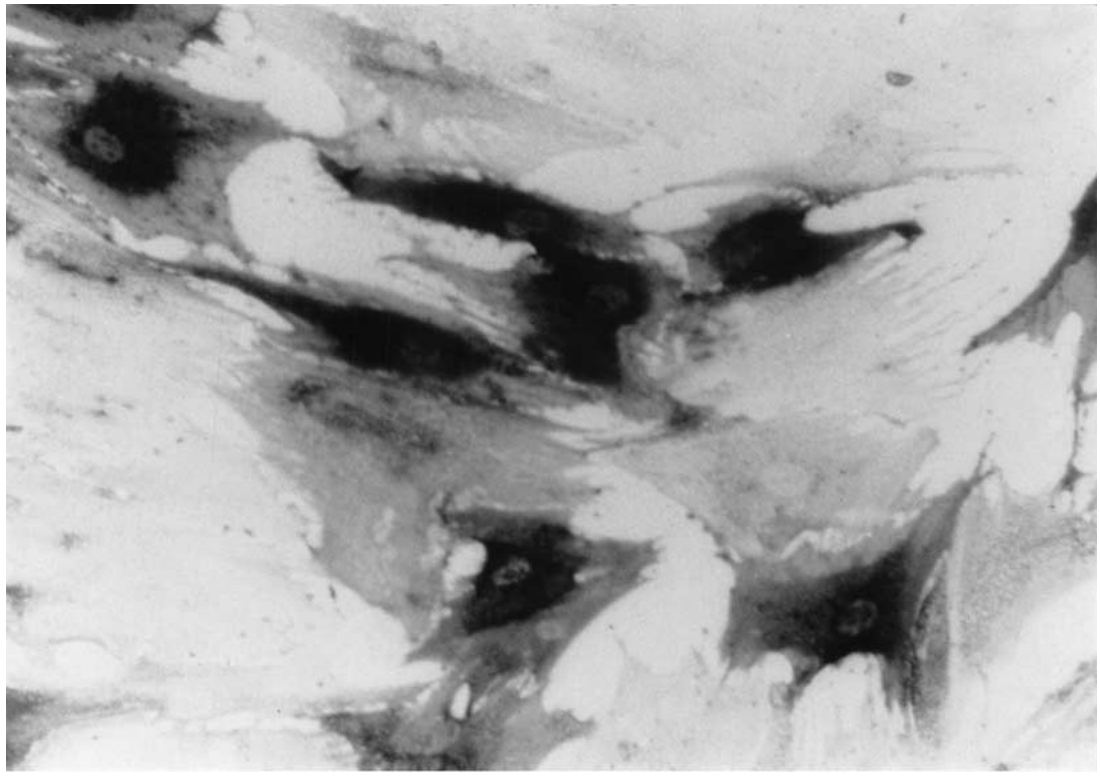

Figure 1 ALP staining was localized in the cytoplasm of osteoblast-like cells (400×) (bar = 25 μm).

matrix when DMEM was supplemented with β-glycerophosphate and ascorbic acid for 7 days, as demonstrated by the positive von Kossa staining for calcium phosphate salts, which appeared as black patches over the culture (Fig. 2).

Positivity for osteonectin and type I collagen (Fig. 3) was expressed in the cytoplasm and in the extracellular matrix. Immunoreactions for type III collagen and fibronectin were also positive.

3.2. Osteoblast on HA samples

3.2.1. Cell growth

Cell growth on the HA substrates was three- to six-fold lower than on control disks (Fig. 4). In particular, on day 28 2141 (±350) cells/well grew on the most porous (group C) disks vs. 12,184 (± 3038) cells/well in the control group, with highly significant differences ($F_{1,4} = 32.3$; $p < 0.005$). At the same time point, 4208 (±954) cells/well grew on group B disks ($F_{1,4} = 18.8$;

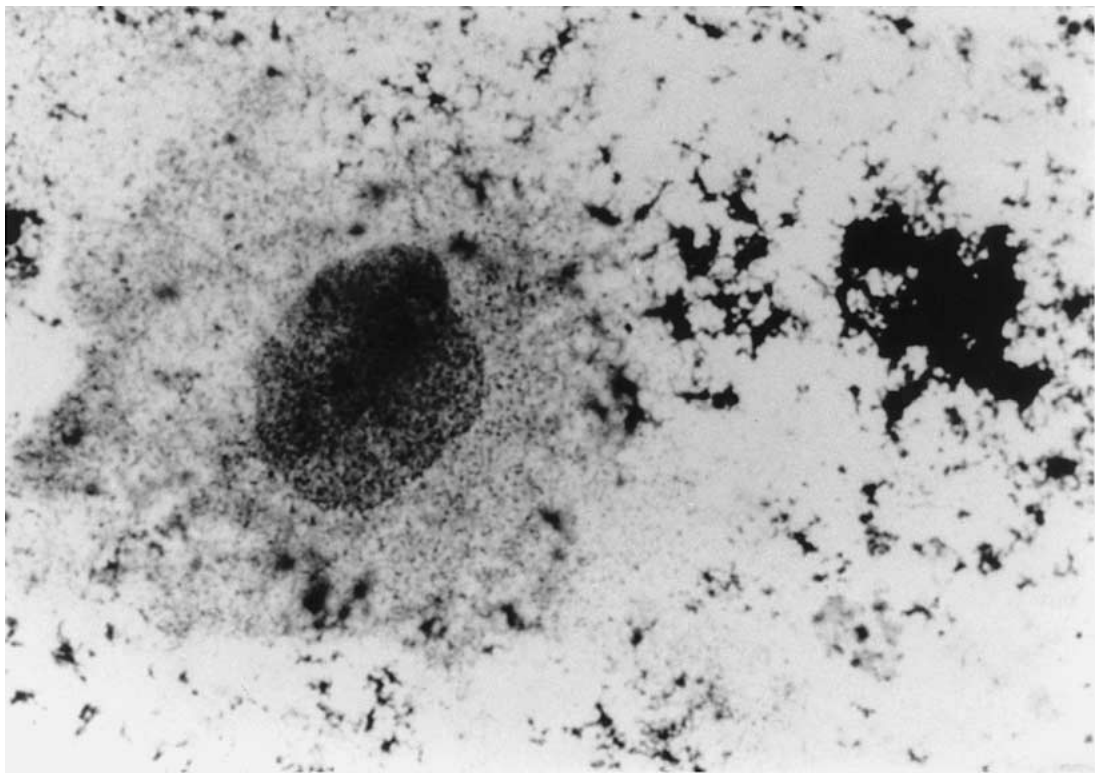

Figure 2 Calcium deposits in osteoblast-like cell cultures showing black reaction product with Von Kossa staining (630×) (bar = 16 μm).

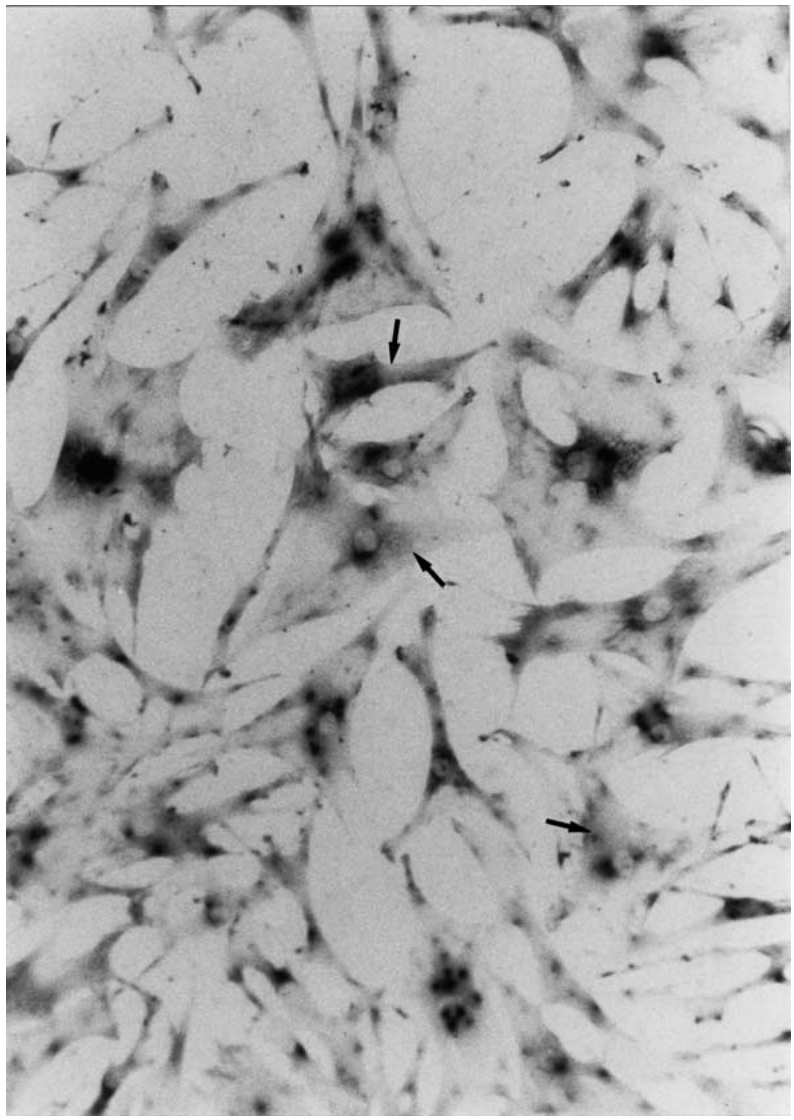

Figure 3 Collagen type I immunoreaction was observed in the cytoplasm of human osteoblast-like-cells (200×) (bar = 50 μm).

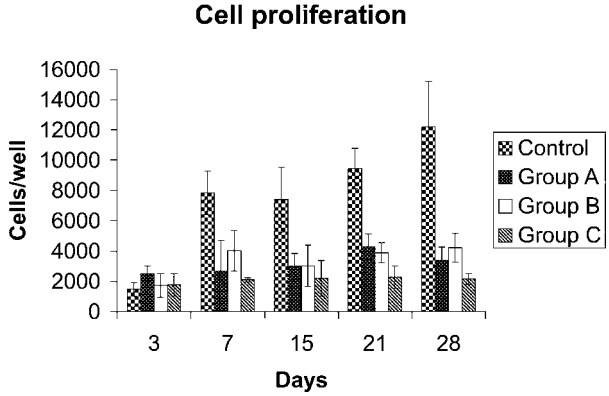

Figure 4 Proliferation rate on HA disks with different porosity : groups A (20%), B (40%), and C (60%). The differences among A, B, C and controls were statistically significant at day 7 ($F_{3,8} = 10.05$; $p < 0.005$), 15 ($F_{3,8} = 7.8$; $p < 0.05$), 21 ($F_{3,8} = 32.9$; $p < 0.0005$), and 28 ($F_{3,8} = 22, 5$; $p < 0.0005$). Significant differences were detected among groups A, B, and C only at day 21 ($F_{3,8} = 32.9$; $p < 0.0005$) and 28 ($F_{2,6} = 5.4$; $p < 0.05$).

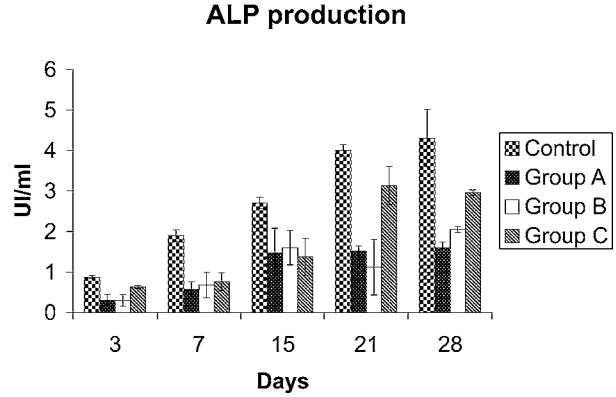

Figure 5 Total ALP production in cultures grown on HA disks with different degree of porosity : groups A (20%), B (40%), and C (60%). Significant differences were observed among A, B, C and control cultures at day 3 ($p < 0.05$), 7 ($p < 0.05$), 21 ($p < 0.05$), and 28 ($p < 0.01$). The differences among groups A, B, and C were significant at day 21 ($p < 0.05$) and highly significant at day 28 ($p < 0.005$).

$p < 0.05$) and 3375 (±875) cells/well on group A samples ($F_{1,4} = 23.2$; $p < 0.01$).

3.2.2. ALP production

ALP production was two- to three-fold lower on HA than on plastic (Fig. 5). On day 28, mean ALP activity was 2.95 (±0.07) IU/well in group C, and did not show significant differences from control cultures ($p > 0.05$); on the less porous disks (groups B and A) mean ALP production was 2.05 (±0.07) IU/well and 1.6 (±0.14) IU/well, respectively (both $p < 0.05$). The evaluation of ALP activity/cell demonstrated major

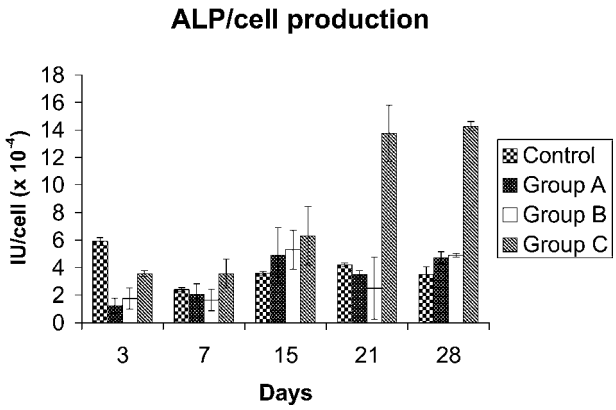

Figure 6 ALP production/cell in cultures grown on HA disks with different degree of porosity: groups A (20%), B (40%), and C (60%). Significant differences were observed among A, B, C and control cultures only after 21 ($F_{3,4} = 23.1$; $p < 0.05$) and 28 days ($F_{3,4} = 269.7$; $p < 0.0005$). The differences among groups A, B, and C were significant at day 21 ($F_{2,3} = 24.7$; $p < 0.05$) and highly significant at day 28 ($F_{2,3} = 465.8$; $p < 0.0005$).

ALP synthesis on all HA disks (Fig. 6), the highest values being observed in group C on day 21 (13.8×10^{-4} IU/cell) and 28 (14.2×10^{-4} IU/cell), with significant differences from controls ($p < 0.05$ and $p < 0.005$, respectively).

3.2.3. Immunofluorescence

In contact with HA, human osteoblast-like cells showed specific immunoreactivity for type I collagen and osteonectin (Fig. 7), with a cytoplasmic and extracytoplasmic pattern.

3.2.4. Scanning electron microscopy (SEM)

SEM analysis allowed to evaluate cell adhesion on HA and to study cell morphology in contact with the material. Osteoblast-like cells had a flattened morphology (Fig. 8) and were often seen to bridge the pores (Fig. 9) and form confluent monolayers. Very few cells showed

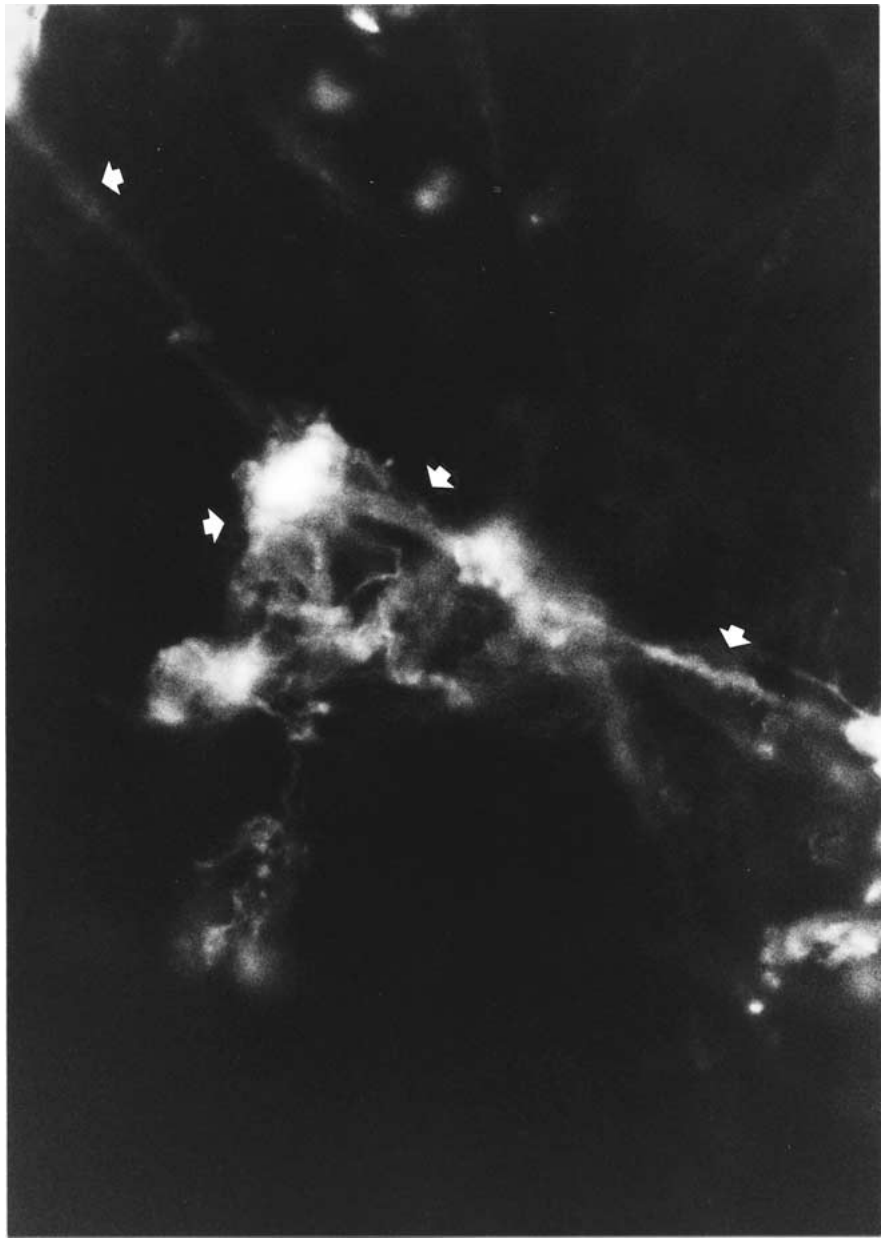

Figure 7 Immunofluorescence for osteonectin was observed in the cytoplasm and extracellular martix of osteoblast-like cells grown on HA (400×) (bar = 25 μm).

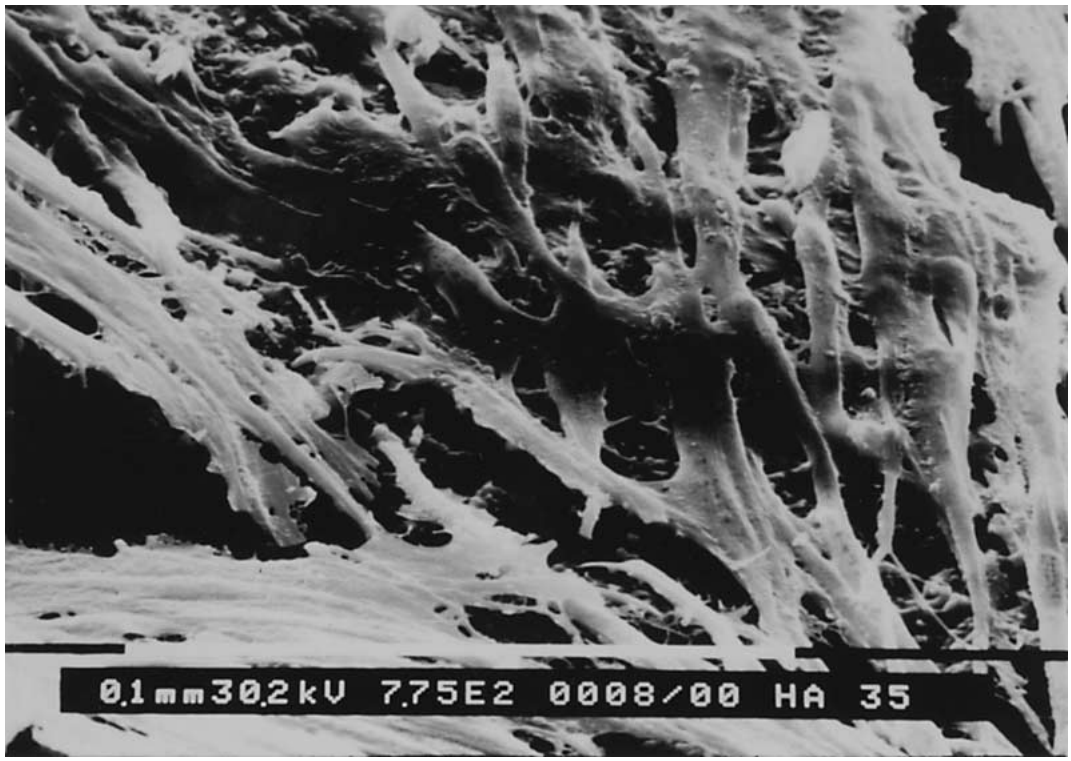

Figure 8 SEM analysis showed osteoblast-like cells growing on HA to have a flattened morphology (775×) (bar = 100 μm).

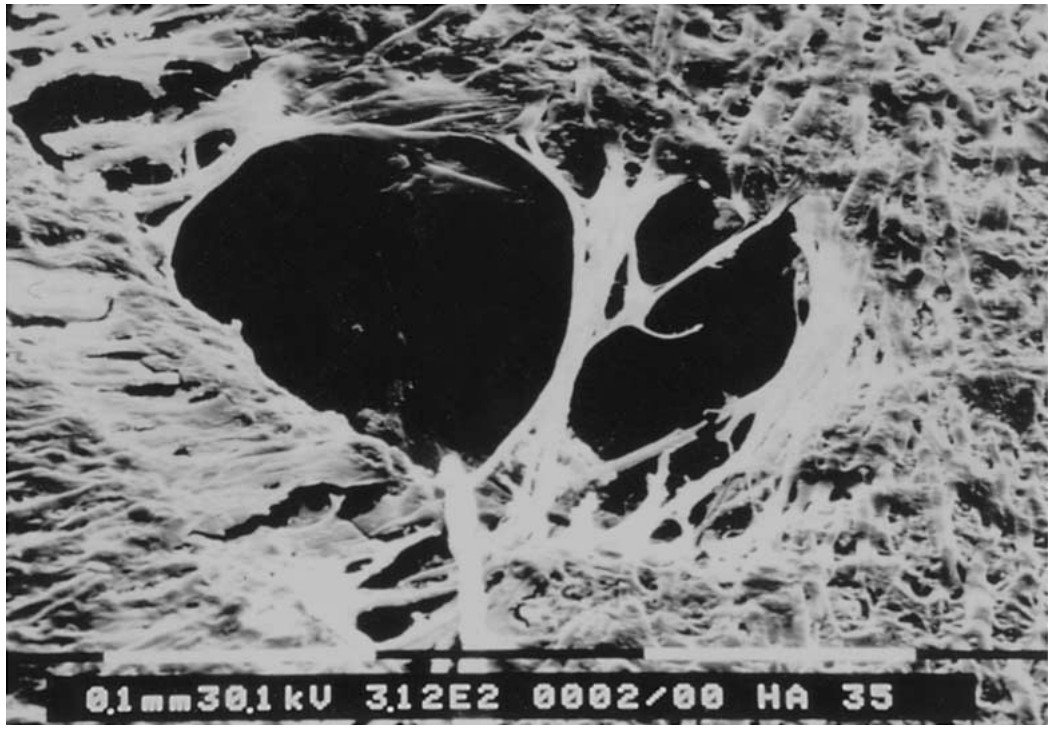

Figure 9 Photomicrograph showing osteoblast-like cells bridging the pores of a low-porosity (20%) HA disk SEM (300×) (bar = 100 μm).

many dorsal ruffles and phylopodia connecting them with the substrate and the other cells.

4. Discussion

We studied cell adhesion, proliferation and differentiation of human osteoblast-like cells grown on HA disks with different porosity (20, 40, and 60%) or on plastic disks to compare the effect of the different degrees of porosity.

To ensure the reliability of this *in vitro* model, we used primary, human, non-transformed osteoblasts [22–24] and characterized them as in previous studies [21, 25]. Osteogenesis induced by osteoblastic cells is characterized by a sequence of events involving

cell proliferation, expression of the marker of the osteoblast phenotype and synthesis, deposition and mineralization of collagenous matrix [26]. A biomaterial study needs to take into consideration all of these aspects. Our results confirm that human osteoblast-like cells can grow on HA [15], but their rate of proliferation was three- to six-fold lower than on the control substrate. In previous studies, cell number was reported to be 28% and 45% lower on HA than on a plastic substrate on days 15 and 28 into culture, respectively [26]. The present study evidenced statistically significant differences in cell proliferation among the HA disks, and higher growth rates on the less porous disks (20 and 40%). We believe that the lower rates observed on the most porous substrate (60%) was caused by the breaking down of the material into fine particles (<5 μm) [27]. Indeed, it has been demonstrated that fine particles of HA, which is normally non–toxic, can cause cell membrane damage *in vitro* [28] due to a direct cell-particle interaction [29].

ALP specific activity, a good index of cell differentiation, was two- to three-fold lower in cells grown on HA than in control cultures. On days 21 and 28, ALP activity was highest in cells grown on the most porous disks and was not significantly different from the activity measured on control cultures. With reference to ALP/cell expression, values after 28 days were even higher and highly significant for group C with respect to control cultures ($p < 0.005$). The ability of HA to enhance osteoblast differentiation is well known [30] and some authors believe it to be related to pore size [31]. The highest ALP activity values and osteocalcin content have both been obtained with a pore size of 300–400 μm, a diameter that seems to favor the formation of osteon-like structures [31].

We observed that on HA disks osteoblast-like cells synthesized typical bone-enriched proteins, like type I collagen and osteonectin, independently of their degree of porosity, suggesting the maintenance of the osteoblastic phenotype. Hott and co-workers [26] have reported higher production of type I collagen per cell in contact with HA than with plastic. The synthesis of bone matrix proteins in contact with HA might explain the bioactivity observed *in vivo* in this material, i.e. its ability to bind directly to bone, whereas titanium, which is bioinert, does not bind directly but establishes a close contact with bone [32].

SEM analysis showed osteoblast-like cells to have a flattened morphology, with few cells looking metabolically active. Several cells were seen to bridge the pores and form confluent monolayers. Previous studies [17] have identified two types of morphology in contact with materials: a "stand-off" morphology [33], with many dorsal ruffles and phylopodia, indicating a better osteoblast condition, and a flattened morphology without ruffles or phylopodia [34]. The flattened morphology of osteoblast-like cells spreading on HA ceramics is already well known [34].

Our data demonstrate that porosity affected the proliferation and differentiation of human osteoblast-like cells *in vitro* and differences became significant at the longer culture times. In particular, the highest degree of porosity was associated with a decrease in cell population and with increased ALP activity, whereas the less porous disks exhibited high proliferation and poor differentiation rates.

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