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Biocompatibility testing of a bovine hydroxy–apatite ceramic material with the use of osteo-progenitor cells isolated from human bone marrow

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Bioactive materials such as hydroxyapatite ceramics are known to show a stable interfacial bond with tissues. The objective of this study was to investigate the behaviour of human bone differentiated cells isolated from bone marrow on the surface of a hydroxyapatite ceramic obtained from bovine spongiosa. Scanning electron microscopy and DNA synthesis analysis assessed by [^{3}H]-thymidine incorporation showed cell colonization of the whole material. Immunological studies using monoclonal antibodies to osteocalcin and osteonectin and cytochemical analysis of alkaline phosphatase activity indicated that these cells did not lose their osteoblastic phenotype after 28 days of culture. Furthermore, this study demonstrates the *in vitro* interface between the material and human cells, which is reproduced *in vivo*.

1. Introduction

Hybrid artificial materials, also called bioartificial organs, result from a newly merging concept in which it is possible to build *in vitro* functional units made of artificial materials and living cells [1]. The evolution of biomaterials science can be observed in the increasing part taken by materials of biological origin in the making of biocompatible surfaces which are able to actively cooperate with living tissues and their own cells. Cell culture investigations play an increasing role in biomaterial research as they participate not only in the experimental evaluation of biocompatibility [2] but can also participate in the elaboration of materials which are expected to feature better biointegration and functional substitution [3].

Hydroxyapatite is known to be biocompatible and new bone can directly bond to this ceramic surface. Therefore, these materials have been categorized as bioactive materials [4].

Hydroxyapatite bone substitutes used in orthopaedic or maxillofacial surgery are generally produced by chemical synthesis or derived from natural biological substances modified by physico-chemical treatments [5, 6]. The cell culture systems most frequently used to test bone substitution materials are either osteoblast-like cells (MC3T3-E1) or osteoblast like directly isolated from mice and rats [7–9].

The objective of this study is to investigate the behaviour of human bone cells on the surface of a hydroxyapatite ceramic obtained from bovine spongiosa (ENDOBON®). The human bone cells used in this work are selected osteoprogenitor cells arising from human bone marrow [10], the biological compartment with which the bone substitution material will be confronted *in vivo*.

This biological evaluation indicates that cell colonization, assessed by scanning electron microscopy and [^{3}H]-thymidine incorporation, occured in this material without loss of osteoblastic phenotype expression of the cells.

2. Materials and methods

ENDOBON® (Merck Biomaterials) is a hydroxyapatite ceramic obtained from bovine bone. For the study, all samples were cylindrically shaped (15.4 mm in diameter, 2 mm thickness) and heat sterilized at 120 °C for 2 h.

Osteoprogenitor cells were isolated from human bone marrow stromal cells [10]. Human bone marrow was obtained by aspiration from the iliac crest of healthy donors undergoing hip prosthesis surgery after traumatic shock (20–30 years). Cells were separated into a single suspension by sequentially passing the suspension through syringes fitted with 16, 18 and 21 gauge needles, and plated into 35 mm dishes in IMDM medium (Gibco) supplemented with 10% (v/v) fetal calf serum (Gibco). Osteoblast precursors were isolated by cell cloning followed by successive subculturing until the highest cellular alkaline phosphatase activity was reached.

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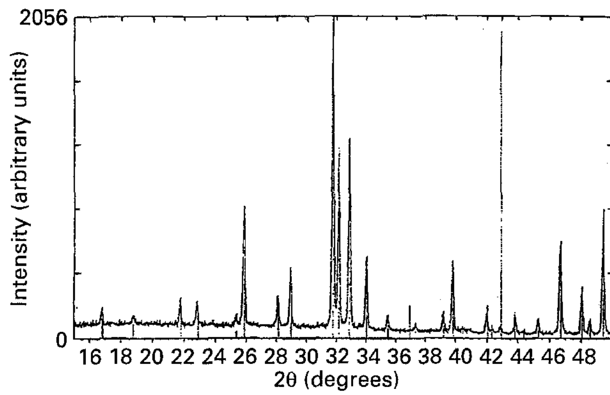
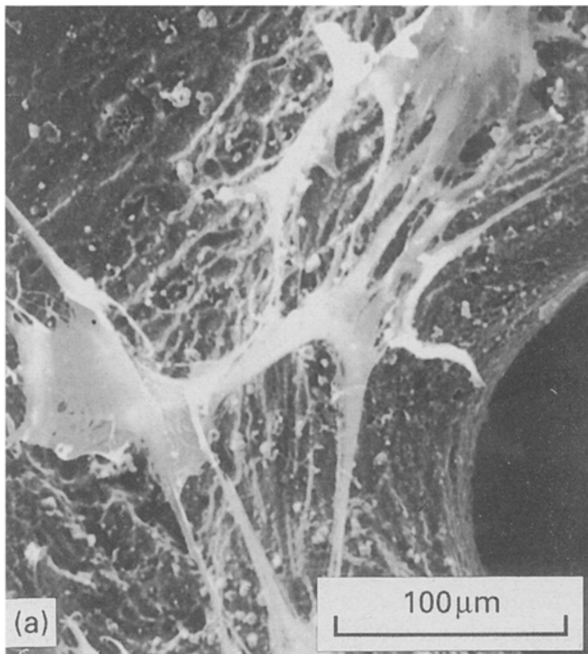
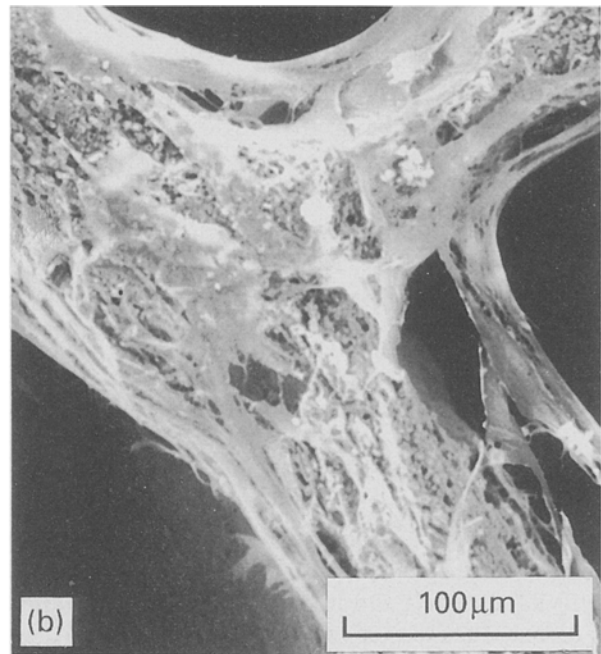


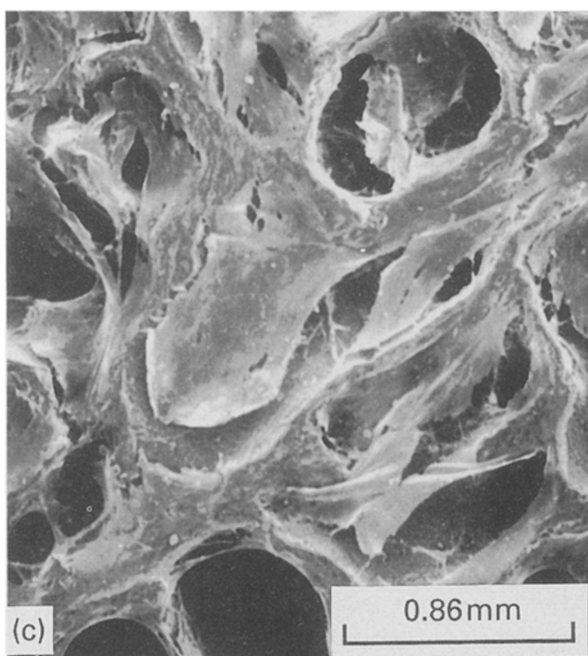
Figure 1 The ENDOBON® ceramic material: X-ray diffactometry.

Cell innoculation on ENDOBON® material was performed as previously described [11]. One day before, agarose gel 2% (w/v) in PBS 0.1 M pH 7 was prepared in four-well culture plates and the samples were placed in each well. Thereafter, concentrated marrow cells (30 000 cells/well) were seeded directly onto the tested material. The culture medium (IMDM) supplemented with 10% (v/v) fetal calf serum was changed every 4 days. The cells adhered onto the materials were investigated by the following methods:

1. *Scanning electron microscopy (SEM)*: performed on days 1, 6, 18, 27 as follows: samples were fixed for 15 min with 2% (v/v) glutaraldehyde in 0.15 M caco-







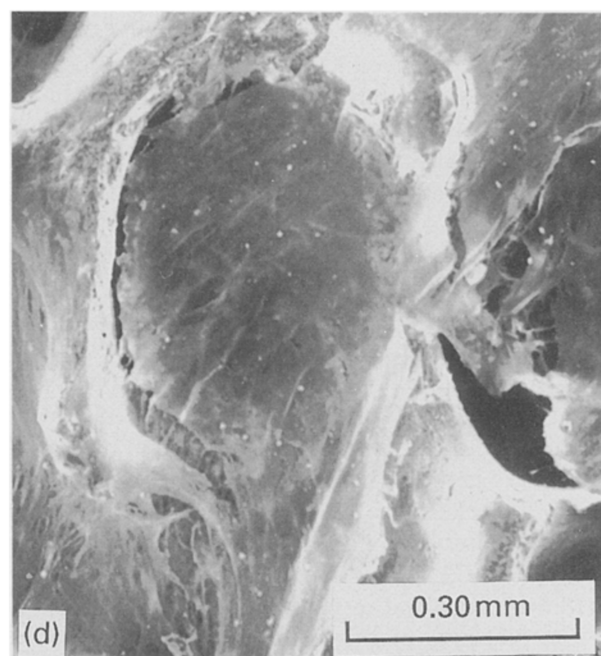
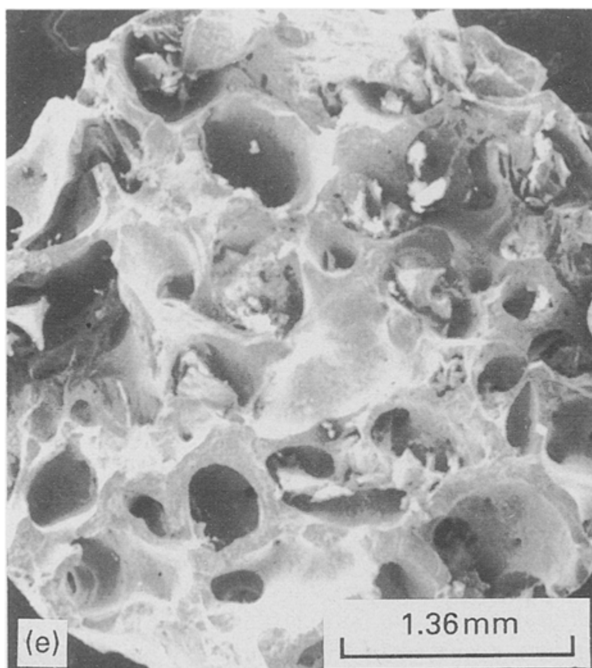
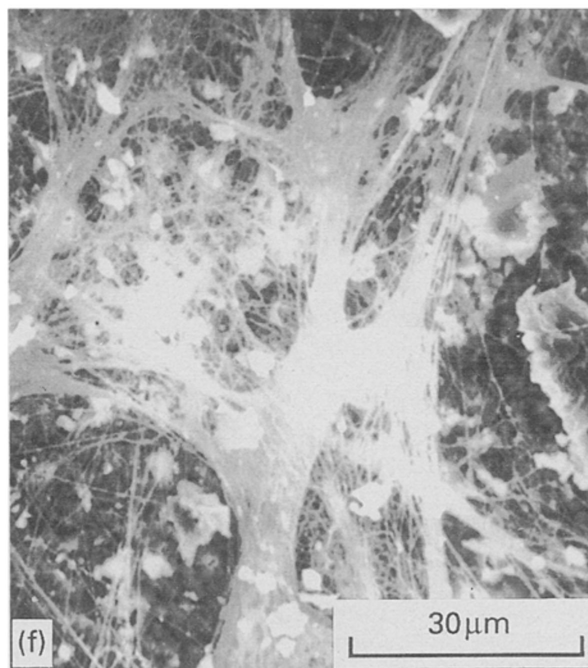


Figure 2 Scanning electron microscopy microgram of ENDOBON® ceramic: osteoprogenitor cells have been cultured for 6 days (a) 12 days (b) 22 days (c), 31 days (d). (e). (f) SEM of a section of the material after 12 days of culture.





dylate for 10 min. Samples were then dehydrated and dried using the critical point CO_2 method and finally coated with metal, with a gold target, before observation with a Hitachi S2500 microscope.

2. *DNA synthesis*: determined by incorporation of [^{3}H]-thymidine according to Hauschka [12]. Cells implanted into ENDOBON® were deprived of fetal calf serum for 24 h and then incubated with [^{3}H]-thymidine (5 µCi/ml) in medium culture as described above. Trichloroacetic acid precipitate was solubilized in 0.2 ml of 0.3 N NaOH and the radioactivity was determined in a liquid scintillation counter (Packard Instrument). Controls were performed using the materials alone.

The expression of osteonectin and osteocalcin was estimated using monoclonal antibodies provided by Cis BioIndusrie (France). Trypsinized cells from material were plated in Lab-Tek Chamber Slide (Nunc), fixed using 100% methanol, and then incubated for 2 h with the corresponding diluted antibodies. After washing with PBS-Tween, fixed immunoglobulins were revealed using a monoclonal PAP detection kit (DAKO).

Osteocalcin synthesis was measured by a specific radioimmunoassay using a detection kit (Cis Bio-Industrie). Alkaline phosphatase activity was determined by cytochemistry as previously described [10] with the Sigma diagnostic kit (85L-2, St Louis, MO), using naphtol AS-MX phosphate as substrate. Intracellular alkaline phosphatase activity was determined at confluency in scraped and sonicated cells as described by Majeska and Rodan [13].

3. Results

The ENDOBON® ceramic has been characterized as a hydroxyapatite ceramic with a high level of purity (HA concentration higher than 95%) (Fig. 1).

Osteoprogenitor cells were isolated and selected according to their alkaline phosphatase activity as previously described [10], or directly by removal of the nodules in the culture plates and seeding in other plates for amplification. This last method quickly led to a pure cell population which exhibited a high alkaline phosphatase activity which was about 0.5 nmol Pi/30 min/µg protein and express 0.045 ng osteocalcin/µg protein.

This highly differentiated cell population was used to study the cell behaviour with the bovine hydroxyapatite (ENDOBON®) using an agarose layer [11] to prevent eventual migration to the plastic dishes of the culture plate. By scanning electron microscopy performed on days 6, 12, 22 and 31 (Fig. 2), it was possible to observe well attached cells spreading out, proliferating and showing morphological signs of extra-cellular matrix synthesis. Cell colonization was progressively observed on every surface of the ceramic cells with covered the pores of the material (Fig. 2d). SEM of a section of the hydroxyapatite (Fig. 2e) after 12 days of culture showed cell development inside the material (Fig. 2f).

DNA synthesis analysis assessed by [^{3}H]-thymidine incorporation into the cells confirmed this proliferation process. After 10 days of culture we observed a stackening of cell division, which could correspond to the production stage of an extracellular matrix (Fig. 3).

Using monoclonal antibodies specific to osteocalcin or osteonectin (Fig. 4a) it was observed that these cells express their osteoblastic phenotype after 28 days of cell culture. Cytochemcial analysis of alkaline phosphatase activity confirmed this last result (Fig. 4b).

4. Discussion

Cellular attachment, cell proliferation and phenotype expression reported in this study indicate that these

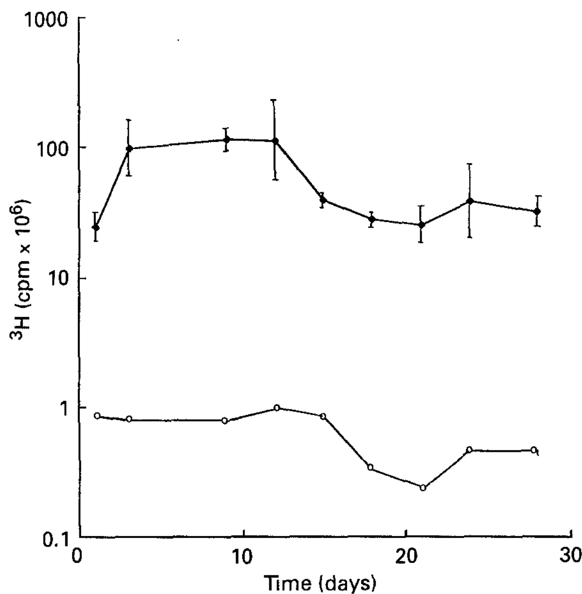


Figure 3 DNA synthesis analysis, performed using [^{3}H]-thymidine incorporation (—○—). Controls (—□—) were performed using only the ceramic (n = 4).

highly differentiated osteoprogenitor cells selected from human bone marrow formed a tissue inside and around the material and did not lose their osteoblastic phenotype.

Scanning electron microscopy performed on a section of the hydroxyapatite shows that these cells are able to colonize the centre of the material. This is confirmed by other authors [14] in an animal model. This bovine hydroxyapatite offered a suitable surface for cell growth and a large area due to the porous architecture (about 90% of the pores have a diameter greater than 100 μm, with a mean size of 450 μm; the pores are interconnected) which allowed a subsequent increase in cell development. This resulted in better bony integration and better interaction with the newly formed bone, although this material is not degradable. Three months after implantation complete bone integration could be observed.

Cell proliferation was confirmed by [^{3}H]-thymidine incorporation. These selected human stromal cells have a rapid growth rate when compared to other materials (i.e. natural coral porites astreoides from Antilles, results not shown). However, after 10 days we observed a stackening of cell division which could correspond to the generation of an extracellular matrix. As we showed by scanning electron microscopy, the cells are no longer limited to growth as a monolayer but can form several supperimposed organized layers. These immobilized cell layers tend to encapsulate the entire HAP material with a mass of neoformed tissue consisting of human stromal bone cells, the matrix synthesized by them and the capture material. The matrix production could correspond to a decrease of [^{3}H]-thymidine incorporation. A comparable result in terms of growth profile was observed with natural coral and fibroblast cells [16], where cell growth occurred in the coral upto the 15th day, reached its maximum on the 18th day and then decreased. Cytochemical and immunological analysis revealed that these cells showed a positive alkaline phosphatase activity, expressed osteonectin and osteocalcin (a bone specific protein) even after 28 days of cell culture, and then demonstrated that they keep a degree of differentiation on the ENDOBON® material, which shows a high level of specific cytocompatibility.

5. Conclusions

In conclusion, the development of hybrid artificial materials for clinical use depends on the cell population which, in order to be useful, must meet certain requirements such as rapid growth rate and a high degree of cell differentiation. Selected human osteoprogenitor cells seem to play a crucial role in bone reconstruction.

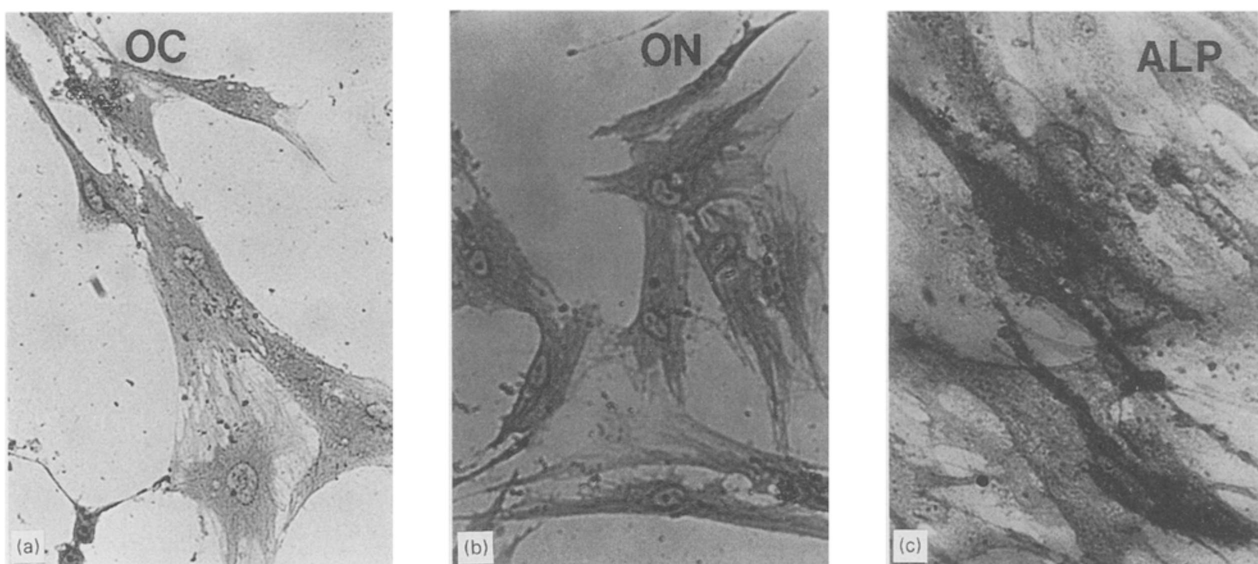


Figure 4 Phenotype expression of osteoprogenitor cells after 28 days of cell culture in the bovine hydroxyapatite ceramic. Immunostaining using monoclonal antibodies against (a) bovine osteocalcin (OC) and (b) osteonectin (ON). Cells were trypsinized from the materials (n = 4) after 28 days of culture and seeded onto chamber slides Lab-Tek (NUNC). Immunological reaction was performed after cell attachment to the plastic dishes. Cytochemical analysis of alkaline phosphatase activity (ALP).

The correct choice of mineral support is also of vital interest. The bovine hydroxyapatite used in this study promotes rapid attachment and cell growth, which consequently leads to an extracellular matrix immobilization.

We hope that human stromal cell immobilization in HAP may be useful as an implantable bioreactor, as well as a possibility to confirm the feasibility of bone cell autografts.

Acknowledgements

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