JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 46–51

Human osteoprogenitor responses to orthopaedic implant: mechanism of cell attachment and cell adhesion

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Cell culture models are becoming prevalent in the investigation of tissue responses to implant materials. Cellular attachment and cell adhesion studies can aid in the development of more effective orthopaedic and dental implants. Cell attachment was studied on extracellular matrix proteins (type I, IV collagen, peptide solubilized elastin (PSE), fibronectin laminin). Human osteoprogenitor cells responded differently to these collagenous and non-collagenous proteins. PSE and type I or type IV collagen are the most effective proteins in cellular attachment and cell spreading. Cell behaviour was measured in the presence of macroporous materials (*Porites astreoïdes* from the West Indies and a bovine hydroxyapatite ceramic ENDOBON®) and bioartificial connective matrices comprising hydroxyapatite, peptide solubilized elastin, collagen, fibronectin and chondroïtin-6-sulfate, components of the extracellular matrix (ECM). Human osteoprogenitor cells responded differently to the materials tested according to the content of components of ECM. About 40% of attached cells were obtained on the composite materials PSE, collagen, fibronectin and chondroïtin-6-sulfate, and about 10% on the macroporous materials, whatever their porosity and their chemical components. These results demonstrate a need for more effective surface treatment to promote cell attachment, cell spreading and cell growth.

1. Introduction

For an implant to be successful, close apposition of bone to the surface is essential and is closely associated with the behaviour of the cells (anchorage, attachment, adhesion, spreading and then cell growth). The formation and deposition of bone directly onto the implant requires a surface that is not only non-toxic but also allows or favours this behaviour. The selection of a suitable surface coating would be facilitated if the nature of the interaction of bone cells with biomaterial surfaces could be predicted from appropriate behaviour of cells in tissue culture [1]. Using a variety of cell types, cell growth and biocompatibility analysis [2–6] were studied on currently used orthopaedic materials. This was carried out particularly on coral [7], hydroxyapatite [4], polymers [8] and on a bovine hydroxyapatite ceramic material [9]. In such a cell culture assay, the secretion of extracellular matrix (ECM) components by the cells on the various materials mediates cell substrate adhesion [10]. A partial list of extracellular matrix products include collagen, osteopontin, laminin, fibronectin, vitronectin. These regulatory effects of ECM are mediated through cell surface adhesion receptors which support the attachment of cells of ECM molecules both *in vivo* and *in vitro* [11, 12].

This work describes a series of experiments designed to examine the cellular responses to different extracellular matrix components and various biomaterials currently used in orthopaedic applications. The human bone cells used in this work were selected osteoprogenitor cells arising from human bone marrow [13] the biological compartment with which the bone substitution material will be confronted *in vivo*.

2. Materials and methods

2.1. Materials used

ENDOBON® (Merck Biomaterials) is a hydroxyapatite macroporous ceramic obtained from bovine bone. For the study, all samples were cylindrically shaped (0.6 cm in diameter, 3 mm thickness). Coral samples (*Porites astreoïdes*) from the West Indies were prepared as 0.7 cm × 0.6 cm × 0.1 cm pieces. ENDOBON® and coral were heat sterilized at 120 °C for 2 h before experiment.

Two types of bioartificial connective matrices elaborated as described previously [14] were also used. The first material is constituted by peptide-solubilized-elastin

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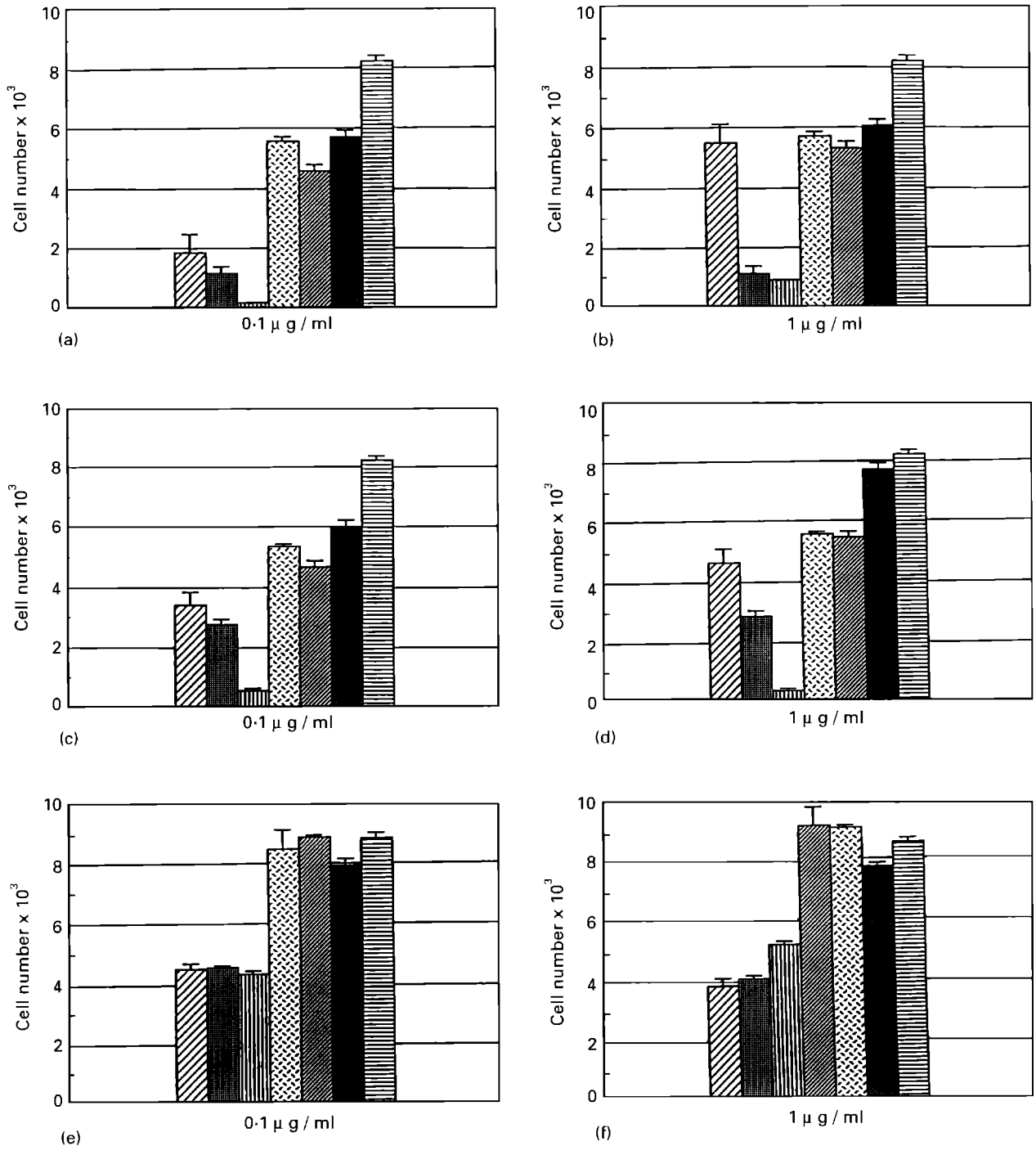


Figure 1 Cell attachment assays on extracellular matrix proteins. Assays were performed using different protein concentrations: 0.1 µg/ml (a, c, e) and 1 µg/ml; (b, d, f) coated onto 96-well culture plates. Human osteoprogenitor cells were incubated for 30 min (a, b), 1 h (c, d) and 2 h (e, f) on coated plates. ▨ fibronectin, ▩ laminin, ▥ BSA, ▦ type I collagen, ▨ type IV collagen, ■ PSE, ▤ plastic. Results are expressed in cell number compared to a standard curve obtained by linear regression of absorbance data of a known cell number.

(PSE), hydroxyapatite (HAP), type I collagen (PSE-HAP-Coll), the second one is supplemented by fibronectin and chondroïtine-6-sulfate (C_6S) (PSE-HAP-Coll-FN-C_6S). The samples were 0.6 cm diameter, 0.1 cm thickness and were sterilized by 15 h irradiation.

2.2. Cell culture model

Osteoprogenitor cells were isolated from human bone marrow stromal cells as described previously [13]. Human bone marrow was obtained by aspiration from the iliac crest of healthy donors undergoing hip prosthesis surgery after traumatic shock (15–60 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 24-well plates in IMDM medium (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco). Osteoblasts were isolated by cell cloning followed by successive subculturing until the highest cellular alkaline phosphatase activity was reached.

2.3. Cell attachment assays

Cell attachment assays were performed as described by Majeska [12] and Landegren [15] with some

modifications for material applications. Extracellular matrix proteins (laminin, fibronectin, type I collagen, type IV collagen and peptide-solubilized-elastin) were diluted in 0.1 M PBS pH 7.4 at different concentrations (10 µg/ml, 1 µg/ml, 0.1 µg/ml) and coated onto plastic dishes for 2 h at 37 °C. With regard to biomaterials studies, to prevent cell attachment to plastic dishes, an agarose layer (2% (w/v) in 0.1 M PBS pH 7.4) was poured into 24-well plates [16]. Thereafter, materials were placed onto agarose layer and preincubated in a medium DMEM (Gibco) without red phenol overnight at 37 °C. Controls were performed using agarose layer alone and plastic alone. A concentrate cell suspension (4×10^5 cells/ml) was directly seeded onto the material or onto the coated wells. The attached cells were quantified by measurement of the lysosomial enzyme N-acetyl-β-D-hexosaminidase using a substrate buffer: p-nitrophenyl N-acetyl-β-D-glucosaminide (7.5 mM of substrate, 0.1 M Na citrate pH 5, Triton 0.5% (v/v)). After washing using 0.1 M PBS pH 7.4, attached cells were incubated for 2 h at 37 °C in humidified atmosphere with the substrate solution. Reaction was stopped by the addition of 80 mM glycine, 5 mM EDTA pH 10.4 and quantified by lecture at 405 nm.

2.4. Scanning electron microscopy (SEM)

Samples were fixed for 15 min with 2% (v/v) glutaraldehyde in 0.15 M cacodylate for 10 min. Samples were then dehydrated and dried using critical point CO_2 method and finally coated with metal, with a gold target, before observation with a Hitachi S 2 500 microscope.

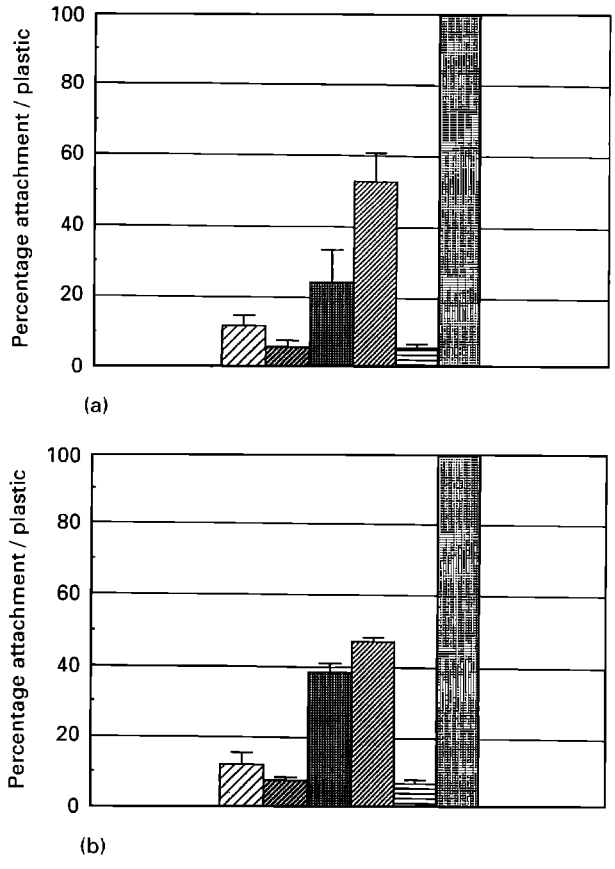


Figure 2 Cell attachment assays on biomaterials. Cells were seeded onto biomaterials, ENDOBON® (▨), *Porites astreoïdes* (▨), the bioartifical connective matrices HAP-PSE-Coll (■) and HAP-PSE-Coll-FN-C_6S (▨) as described in Materials and methods and cell attachment assays were performed after 1 (a) and 2 (b) hours of incubation. Controls were performed using agarose alone (▤) and plastic dishes (■). Results are expressed in percentage of attachment when compared to the results obtained with plastic.

3. Results

When compared to the results obtained using fibroblast cells (not shown), cells expressing an osteoblast phenotype do not exhibit the same behaviour towards the different extracellular matrix protein coated wells (Fig. 1).

As concerns fibronectin-coated wells, cells seem to be more sensitive to the protein concentrations than to the incubation period. Maximum attachment is observed for 1 µg/ml of fibronectin after 30 min of cell incubation (Fig. 1b); 50% attached cells were obtained in this condition. However, for lower concentrations (0.1 µg/ml), an incubation period of 2 h (Fig. 1e) is effective for obtaining a comparable cell attachment. The cell response to laminin indicates that cell attachment increases only with the incubation period. A maximum response is obtained after 2 h incubation (Fig. 1e and f), whatever the concentration used. A similar response is observed with bovine serum albumin which could indicate a non-specific reaction of the laminin protein. With regard to collagenous proteins (type I or type IV), comparable results are obtained with the two concentrations studied, the maximum response is obtained after 2 h cell incubation. Furthermore, in these conditions, cell attachment to type I or type IV collagen is about the same as that observed for plastic, which demonstrates the efficiency of collagen coating in cell attachment and cell adhesion process. Cell behaviour towards PSE is the same as observed with collagenous proteins. The lowest concentration (0.1 µg/ml) is as effective as the highest (1 µg/ml), and a 2 h incubation period also permits maximum cell attachment in comparison with the plastic.

One part of these results could explain the difference in cell attachment and cell adhesion to the different orthopaedic materials tested (Fig. 2). The composite material HAP-PSE-Coll promotes high cell attachment (Fig. 2a) after 1 h cell incubation (22%). The addition of glycosaminoglycan, the chondroïtin-6-sulfate and fibronectin enhances this process (53%). Although 2 h cell incubation increases the percentage of attached osteoprogenitor to the first material (HAP-PSE-Coll) (38%), this incubation period did not modify significantly the percentage of attached cells to the second composite material (HAP-PSE-Coll-FN-C_6S).

Whatever the macroporous materials studied, the coral *Porites astreoïdes* (Fig. 2b), or the bovine hydroxyapatite ceramic (Fig. 2c), comparable results were obtained even after 2 h incubation. 10% of the cell attachment to the plastic dishes was obtained for both materials.

Scanning electron microscopy (Fig. 3) performed for both of the macroporous materials, shows that

after 1 h, *Porites astreoïdes* cells appeared still rounded, while after 6 h, cells exhibit long cytoplasmic extensions with focal anchorage spot.

4. Discussion

In this study, we demonstrate that the adhesive properties of human osteoprogenitor cells differ according to the substrate. These cells exhibit similar patterns of attachment to collagenous proteins and peptide-solubilized-elastin which are the most effective substrates for cell attachment, even for the lowest concentrations. An eventual saturation process of the surface by the collagenous proteins or by PSE could be elucidated by the use of lower concentrations (0.01 and 0.001 µg/ml).

Fibronectin yielded the highest fraction of attached cells in the assay performed using 1 µg/ml of fibronectin and 30 min cell incubation. Neither a longer cell incubation period nor use of the highest concentration of fibronectin (10 µg/ml) (not shown) modified the proportion of attached cells.

In contrast to collagen proteins to PSE or to fibronectin, osteoprogenitor cells exhibit a lower affinity to laminin-coated wells, whatever the concentration tested.

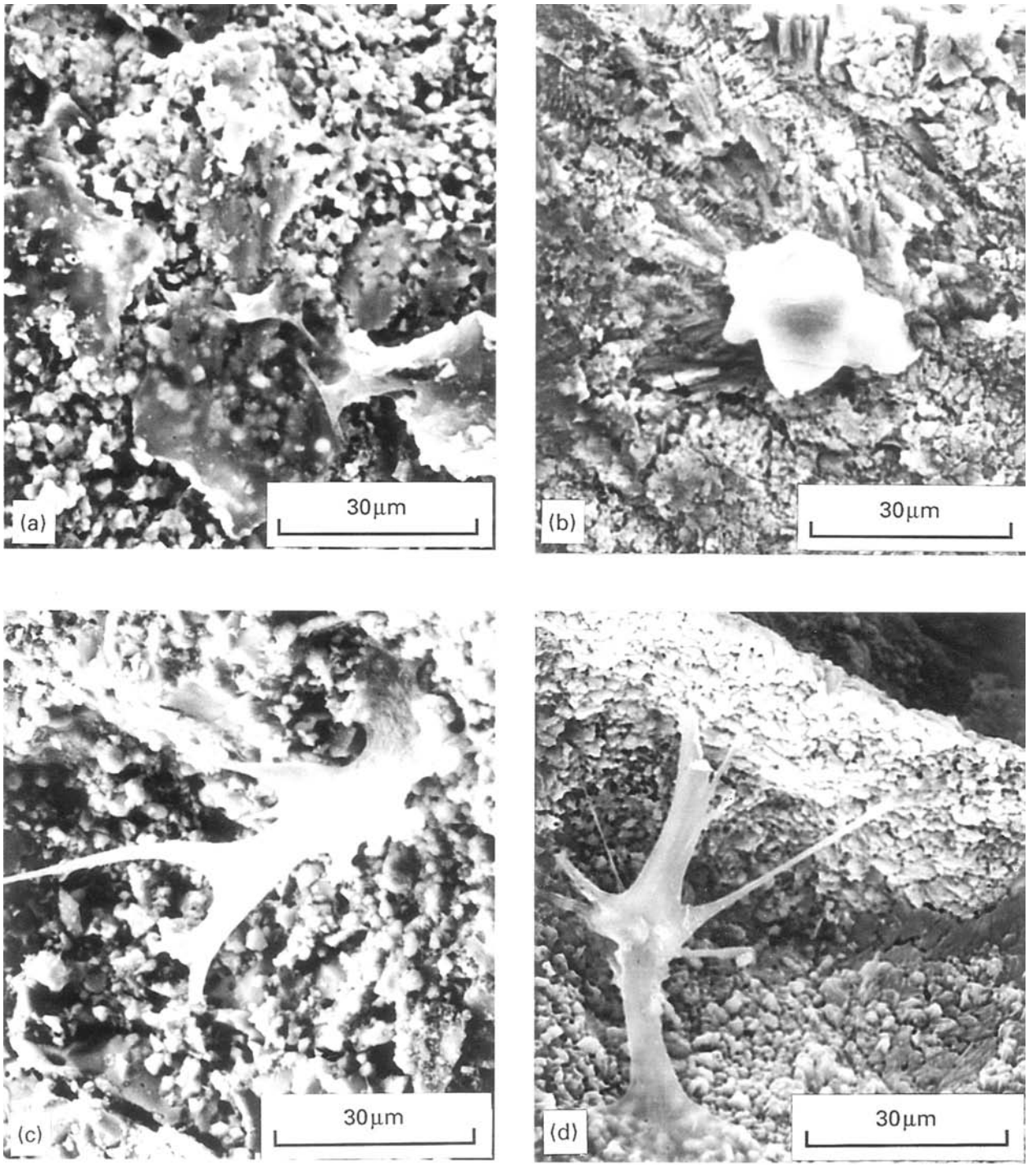
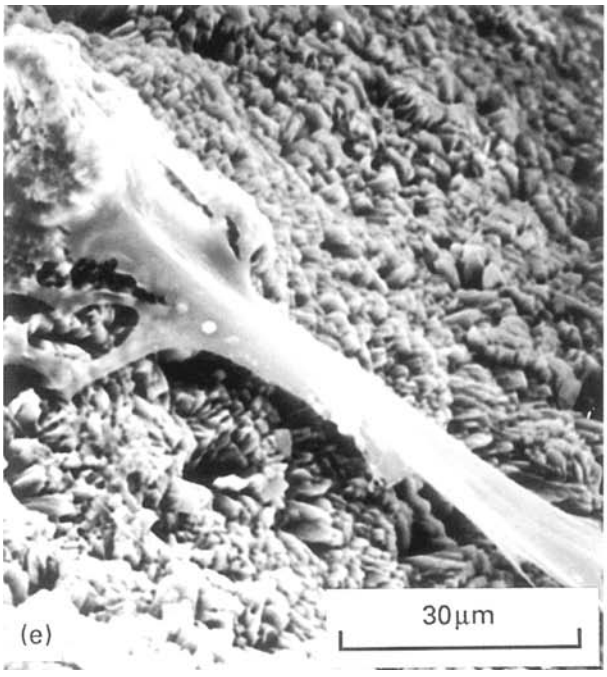
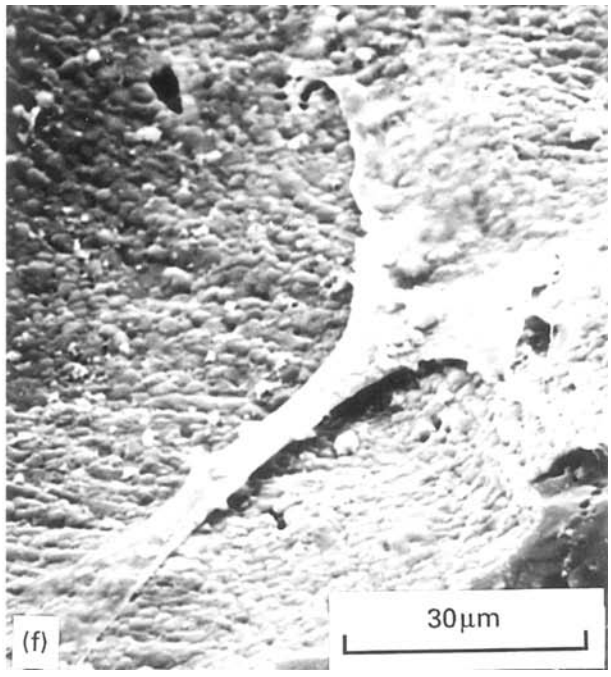


Figure 3 Scanning electron microscopy of osteoprogenitors on the biomaterials. SEM was performed on ENDOBON" (a, c, e) and *Porites astreoïdes* (b, d, f) after 2 h (a, b), 6 h (c, d) and 24 h (e, f).





The finding that attachment of osteoprogenitor cells is more effective with collagen, fibronectin and PSE coatings is related to the results obtained with the artificial connective matrices. Comparative studies performed on the two types of composite materials, HAP-PSE-Coll and HAP-PSE-Coll-FN-C_6S show the highest fraction of attached cells on the latter. Moreover, the same proportion of attached cells onto HAP-PSE-Coll-FN-C_6S is obtained for both the two incubation periods (between 1 and 2 h). The difference in these two studied connective matrices is the presence of fibronectin, a major constituent of ECM, and a glycosaminoglycan, the chondroïtin-6-sulfate. Cell attachment assays were not performed using chondroïtin-6-sulfate coating, but the results obtained using fibronectin seem to predict its function in the cell adhesion process in this biomaterial. Moreover, whatever the cell incubation period (1 or 2 h) the same proportion of attached cells is obtained onto HAP-PSE-Coll-FN-C_6S. As we can show concerning ECM coatings, the fibronectin effect is maximal for the early incubation period (30 min), which could explain the effectiveness of the cell attachment process of the second connective matrix (HAP-PSE-Coll-FN-C_6S) at 1 h cell incubation.

The organization of these extracellular matrix proteins into the hydroxyapatite phase is unknown. Microstructural analysis carried out on the upper face of the material by scanning electron microscopy revealed a high concentration of collagen fibres on the hydroxyapatite granules (Fig. 4). These fibres probably enhance the attachment and the cell adhesion mechanism as observed earlier using wells coated with type I collagen (Fig. 1).

The lower face which contains concentrated hydroxyapatite granules does not possess the same surface characteristics, which probably induces different cell attachment.

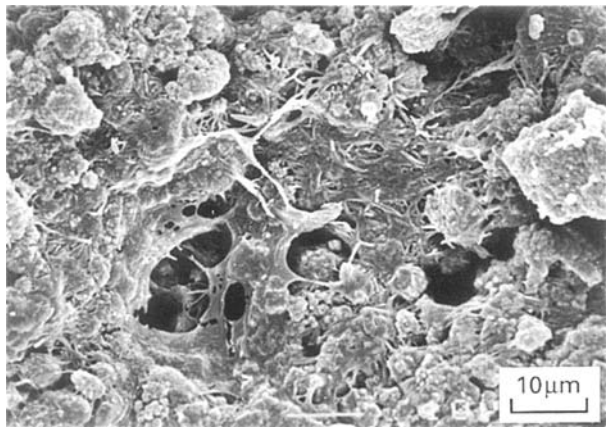


Figure 4 Scanning electron microscopy of bioartificial connective matrix.

The second group of materials studied are macroporous materials. Although they differed in their chemical components (calcium carbonate for *Porites astreoïdes* and calcium phosphate for ENDOBON®) and in their porosity, which are, respectively, about 150 µm pore size and 400 µm to 1500 µm for coral and bovine hydroxyapatite, the percentage of cell attachment is the same in both materials. The difference in cell behaviour towards these materials could appear during the following steps of cell colonization (cell growth and cell differentiation).

Scanning electron microscopy performed at different times on the two macroporous materials revealed cellular spreading which generally occurred during the first 120 min after cell seeding. This result is confirmed by Howlett *et al.* [17]. Thereafter, the cell/biomaterial interaction is followed by a cellular adhesion which involves the secretion and assembly of an extracellular matrix.

In conclusion, the results obtained in this study justify further investigations into the cell adhesion process particularly in the function of well-known adhesion molecules [12] ($\alpha_3\beta_1$, $\alpha_V\beta_1$ integrin ...) identified previously in osteoprogenitor cells (not shown) by enzyme-linked immunosorbent assay.

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Received 29 June
and accepted 4 July 1995