JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE, 4 (1993) 575–581

Natural coral as a substrate for fibroblastic growth *in vitro*

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Coral skeleton is a naturally porous material. This biomaterial is nowadays currently used in humans as a bone graft substitute. Its open porosity provides a large area for potential cell adhesion and for this reason fibroblastic cell proliferation into this material was investigated. McCoy's human fibroblasts were cultured on coral specimens; cell viability and propagation were evaluated by the means of an MTT test and manual counting. Due to the biomaterial porosity, numerous technical adjustments were necessary to ensure an acceptable cell development and evaluation of cell populations. Cell population was evaluated every 3 days over a 24-day period. When compared to cell growth on culture membranes, fibroblasts grew slower on coral during the early time periods, but increased to 2.2 times that of controls from the 12th day after seeding. Natural coral seems to offer a suitable surface for large fibroblastic culture and could be an interesting alternative to synthetic substrates traditionally used for this purpose.

1. Introduction

The benchmark for bone replacement is bone autograft. While autografts are clearly osteogenic, there is a limited supply of bone and the need for a second operation to harvest the graft adds a degree of morbidity. For this reason the field of bone substitutes has been widely investigated during the past decade. There is a great selection of biomaterials to choose from, depending on the surgical needs. A very complete review on this subject has been published by Damien and Parsons [1].

One material currently used as a bone graft substitute in Europe is natural coral, which consists of calcium carbonate ($CaCO_3$) skeletons in the form of aragonite. Animal experiments carried out by Guillemin and colleagues [2] have demonstrated (i) initial invasion of the coral structure by bone marrow elements accompanied by blood vessels, followed by (ii) osteoclastic resorption and (iii) osteoblastic bone formation. A key role is played by carbonic anhydrase, an enzyme present in osteoclasts. Coral resorption and newly formed bone were found to be related to (i) animal species, (ii) coral porosity and (iii) implantation time [3]. There was a gradual transformation of the crystalline structure of aragonite to poorly crystalized apatite [4, 5]. These properties of biocompatibility, resorbability and substitution by host bone have made natural coral a biomaterial useful for filling small and medium size bone defects. In some cases of large bone loss the grafted site seems to undergo an osteogenic slackening, leading after some weeks to a lack of repair. In order to overcome this weakness, a cell-preinfiltrated biomaterial was studied. For this, it was necessary to design a protocol to study fibroblastic growth on porous calcium carbonate. Section 3 consists of modifying currently used *in vitro* cell culture procedures for growth of fibroblasts on coral. Once this was accomplished a standard procedure was used to determine whether or not: (i) the surface of natural coral represented a good support for *in vitro* fibroblastic adhesion, compared to treated cell culture membrane; and (ii) the considerable increase of surface area due to the porous architecture of the coral used, led to a proportional increase in cell population.

Thus to optimize cell growth and its evaluation in the three-dimensional architecture of coral, two successive experiments were necessary. The *in vitro* techniques for use in the study of natural coral as a cell support are modified in Section 3. *In vitro* study of cells on coral substrates were quantitated and analyzed using SEM in Section 4.

2. Materials and methods

The assay utilized in this experiment to measure cell growth used a 1 mg ml^{-1} MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution of medium, without red phenol or FCS, a 3 h incubation time and pure isopropanol as the solvent. In all experiments the coral used belonged to the genus *Porites* sp. Porosity volume was evaluated at 49 $\pm$ 2%. Mean pore diameter, as well as the trabecular cross-sectional diameters, was *ca* 250 μm (range 100–350). This calcareous biomaterial consisted of 98–99% calcium carbonate in the orthorhombic crystalline form, termed aragonite. The remaining 1–2% was made up of various amino acids and oligoelements integral to the calcareous skeleton. The porous fenestrated structure maximized the surface area in contact with external elements (Fig. 1).

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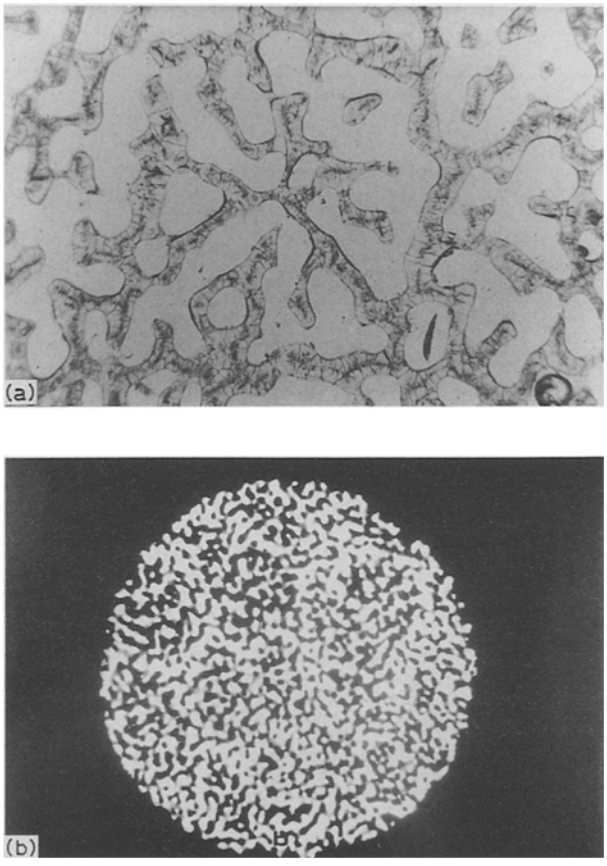


Figure 1 Coral surface. (a) Optical microscopic view, ×60. (b) Microradiograph of the same specimen.

All the specimens were shaped on a high-speed lathe using diamond tools and sterilized by ionizing radiation (2.5 Mrad).

The cell line chosen for study was the McCoy's cell line (Flow Laboratories, Irvine, UK), which consists of human synovial fluid fibroblasts. These were maintained in M199 medium supplemented with 10% foetal calf serum (FCS) and incubated in a humidified atmosphere at 37 °C and 5% gaseous CO_2 tension.

3. Modification of *in vitro* techniques for use in the study of natural coral as a cell support

In order to quantitate cell viability and to evaluate the number of cells, the MTT test was used. The MTT test was developed by Mosmann [6] and represents a quantitative semi-automated staining method for evaluation of viable cell populations. The amount of tetrazolium salts corresponds to the activity of various dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria, producing formazan, thus the reaction occurs only in living cells.

Mosmann [6] developed this method to assess cell proliferation and cytotoxic effects of various lymphokines. An MTT solution concentration of 5 mg ml^{-1} was added to supplemented 10% FCS medium and incubated for 4 h at 37 °C. Then a solvent (acid–isopropanol) was added to dissolve the dark blue crystals of formazan. The solution of salts was analysed in a spectrophotometer through a 630 nm wavelength filter.

In order to improve the method, Denizot and Lang [7] incubated cells for 3 h directly in a 1 mg ml^{-1} MTT solution. Medium without serum and with pure ethanol as the solvent was used to avoid precipitation problems. Medium without red phenol was also used to overcome alteration of the spectral properties of formazan salts. The formazan solution was read through a 560 nm spectrophotometer filter.

To compare the drug sensitivity of different cell lines. Carmichaël *et al.* [8] used clonogenic and MTT assays as well. Cells were incubated for 4 h in a 2 mg ml^{-1} MTT solution added to medium supplemented with 10% FCS. Better absorption characteristics were observed when DMSO (dimethylsulphoxide) was used as the solvent. The solution of salts was read through a 570 nm spectrophotometer filter.

Sgouras and Duncan [9] evaluated the biocompatibility of soluble synthetic polymers by means of an MTT test. They obtained similar data when they compared a modified MTT test with routine analyses generally used for viability and growth of cell cultures, including incorporation of radiolabelled nucleotides or amino acids and measurement of cell numbers. They used a 5 mg ml^{-1} MTT solution added to the medium 5 h before the end of the incubation period. Medium was removed, insoluble formazan crystals were dissolved in DMSO and the absorbance was measured in a spectrophotometer through a 550 nm filter.

3.1. Experimental procedure I

Cells were cultured on coral plates in one-side flattened tubes (Nunc 156758). Rectangular coral plates measuring 20 mm long, 10 mm wide and 1 mm thick were used. Sixty tubes, 30 with and 30 without a coral plate, were seeded with 2.4×10^5 cells in 3 ml of medium. The flat area of the tube without coral was used as a control. Cell growth in six coral specimens and six controls was measured every two days for 10 days. Medium was replaced every two days.

Cell growth was evaluated by the means of an MTT test. Optical density values were measured in a spectrophotometer (Elisa, Dynatech) at 550 nm wavelength with a 630 nm reference filter.

In order to quantify the true cell growth in the coral without any side-effects from the cells adherent to the plastic, the cell-infiltrated coral was placed in a new container at day two. Cell growth in the coral and in this new container were evaluated separately.

3.2. Results I

Results of this initial trial demonstrated good growth on the control surfaces but relatively unsatisfactory growth on the coral (Fig. 2). It was thought that the poor infiltration of the porous coral by the medium was responsible for this situation. Consequently it was decided to improve this parameter through a change in the initial procedure.

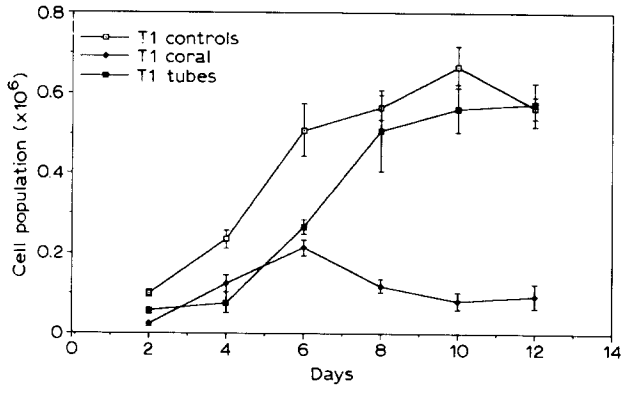


Figure 2 Experimental procedure I (T1). Coral specimen are not pre-infiltrated. Normal growth is observed on plastic surfaces (T1 controls and T1 tubes), while unsatisfactory cell growth is observed on coral surfaces (T1 coral).

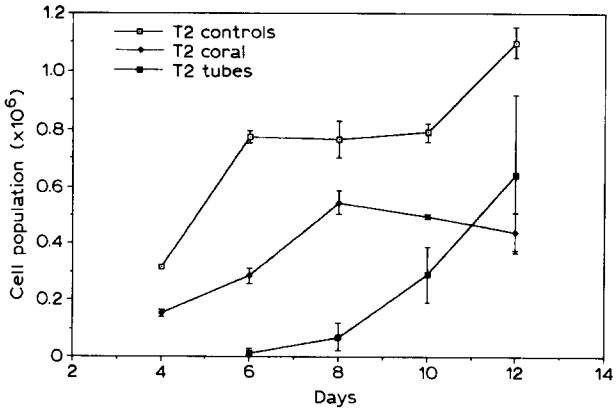


Figure 3 Experimental procedure II (T2). Coral plates are progressively pre-infiltrated with medium supplemented with 10% FCS. This second trial shows an overall increase in the number of cells grown on the coral plates (T2 coral) compared to T1. However, values are still only about 50% of those seen on the plastic controls (T2 controls). Compared to T1 the number of cells which have migrated from coral to plastic tubes (T2 tubes) has decreased remarkably.

3.3. Experimental procedure II

To remove air trapped in the pores of coral it was necessary to treat the specimens as follows: (i) washing in distilled water for 1 h; (ii) sonification for 15 min; (iii) infiltration with hot water (70 °C) under light vacuum (0.013 MPa) for 30 min; (iv) sonification for an additional 15 min.

By nature the coral surface is hydrophobic. Thus to help the media supplemented with FCS to fill the pores and to adhere to the surface after the above treatments, the following procedures were applied: (i) infiltration with medium without FCS under light vacuum (0.013 MPa) for 30 min; (ii) sonification for 15 min; (iii) infiltration with medium supplemented with 10% FCS under light vacuum (0.013 MPa) for 45 min.

To ensure that the cells counted were all initially contained in the coral specimen, the cell-infiltrated coral plates were placed into a new container at day four. Except for these additional procedures, the same protocol used in the first step was applied.

3.4. Results II

Results of this second trial showed an overall increase in the number of cells grown on the coral plates (Fig. 3). However, values were still only about 50% of those seen with the controls. A third step was devised to eliminate additional migration from the coral to the plastic support and to enable counting of cells trapped within the pores of the coral.

4. Quantitation and SEM analysis for a third system for *in vitro* study of cells on coral substrates

In the preliminary steps two processes were observed that caused necessary changes in the protocol.

1. Cell migration from the coral to the plastic support was noticed. This phenomenon may have affected the absolute values of the cell counting and a new set-up was designed.
2. Following the colorimetric test, it was found that the vital staining of the cells was trapped in the pores of the coral specimens and that the crystals could not be completely washed out or solubilized. This resulted in a systematic underestimation of the optical density values.

4.1. Experimental procedure III

Specimens utilized were coral discs 25 mm in diameter and 3 mm thick. In order to prevent cell migration, a special glass holder was designed to support the specimen (Fig. 4).

In order to avoid the systematic underestimation in spectrophotometric values due to the staining trapped in the pores of the coral, specimens were infiltrated with isopropanol and crushed in a ceramic mortar until a fine powder was obtained. Centrifugation (2500 r.p.m. for 7 min) was then applied before analysis of the supernatant in the spectrophotometer at 560 nm wavelength.

Thirty-six coral specimens and 36 controls were seeded with 1×10^5 cells suspended in 200 µl of solution onto the upper coralline surface. To ensure cell adhesion on and into the specimen, the system was left to incubate for 3 h without additional medium. Afterwards, to increase infiltration into the pores the final volume of medium was adjusted by drip-feeding up to 9 ml. Controls used were culture inserts (Falcon 353090) with a polyethylene terepthalate (PET) membrane 25 mm in diameter, 0.45 µm pore size, 11 µm in thickness. Inserts and coral discs on holders were positioned into wells of 6 multiwell plates (Falcon 353046).

Cell growth in six coral specimens and six controls was measured every 3 days for 24 days. The medium in the remaining wells was replaced by drip-feeding, also every 3 days.

Statistical analysis was done using the paired Student's *t*-test.

The surfaces of native coral specimens and coral specimens inoculated with fibroblasts for 15 days were examined by SEM. Specimens were rinsed with phosphate-buffered saline, fixed with 2% glutaraldehyde

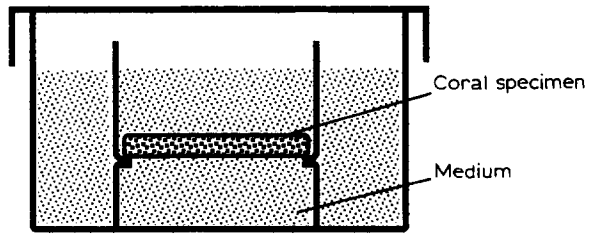


Figure 4 Support for holding the coral specimen.

and rinsed again with the phosphate-buffered saline. They were then dehydrated with acetone and critical-point dried with CO_2, coated with a conductive ultra-thin layer of gold in a sputter coater and examined with a high resolution SEM (Hitachi S800).

4.2. Results III

4.2.1. Calibration of optical density values

Different cell lines show different kinetics of formazan formation, stressing the need for individual calibration curves [10]. So in order to express the optical density values in terms of number of cells, manual counts were performed. A linear relationship was thus established between optical density values and cell populations (Fig. 5).

Six coral specimens and six controls ("inserts") were measured each day up to the 15th day. From this time only coral specimen values were evaluated, due to confluency of cells in the controls. From the 9th day of culture a plateau was reached for the controls and the number of cells did not change dramatically (Fig. 6). The growth of cells in controls was similar to that seen in Section 3.

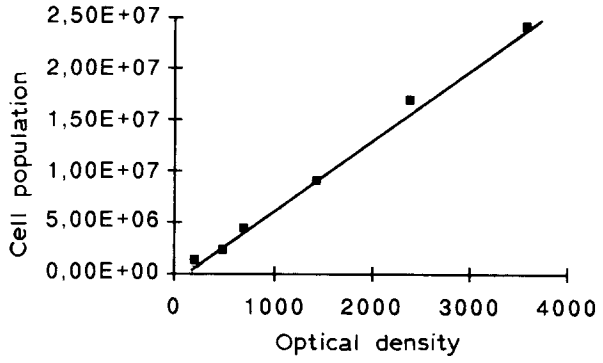


Figure 5 Corresponding values between optical density and manual counts ($r = 0.99$).

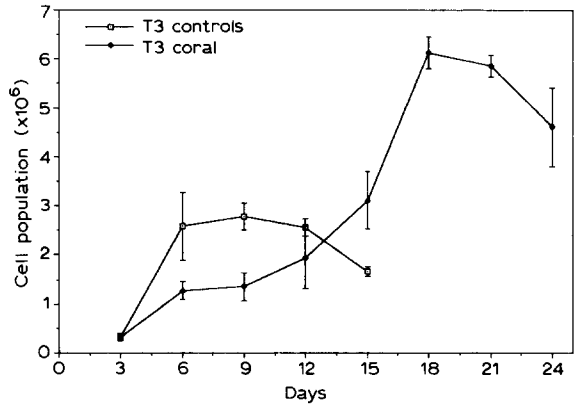


Figure 6 Experimental procedure III (T3). Coral specimens are pre-infiltrated and supported on a special holder during culture. The growth of cells in controls (T3 controls) is similar to that seen in T1 and T2. Compared to the controls, the cell growth in the coral (T3 coral) is slow until the 12th day and reaches a maximum on the 18th day. There is a 2.2-fold increase when compared to the maximum of the controls.

4.2.2. Influence of crushing coral specimens on the MTT assay

Evaluations of optical densities before and after dissolution of formazan salts with pure isopropanol demonstrated a gain of 10.3 ± 5% in cell population value. This gain was significantly different ($p < 0.02$).

Compared to the controls, the cell growth in the coral was slow until the 12th day and reached a maximum value on the 18th day. When compared to experimental procedure II the maximal cell growth in coral specimens was shifted from the 8th to the 18th day. There was a 2.2-fold increase when compared to the maximum of the controls.

4.2.3. Statistical analysis

From the 15th day control values were no longer registered because cell confluency has already been reached at the 6th day. So, results can be statistically analysed for only days 3, 6, 9 and 12. On day 6 control > coral ($p < 0.05$); on day 9 control > coral ($p < 0.01$); on day 15 coral > control ($p < 0.01$).

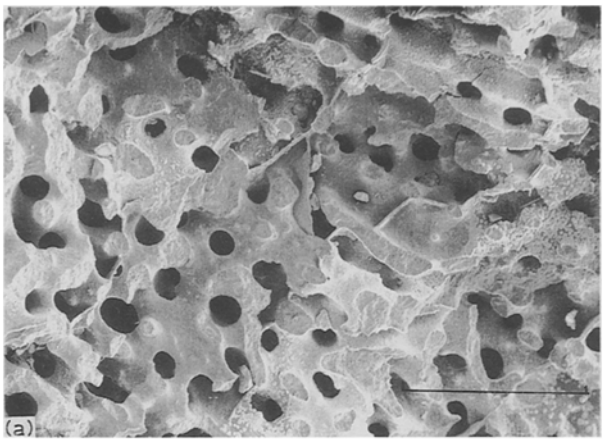
4.2.4. SEM

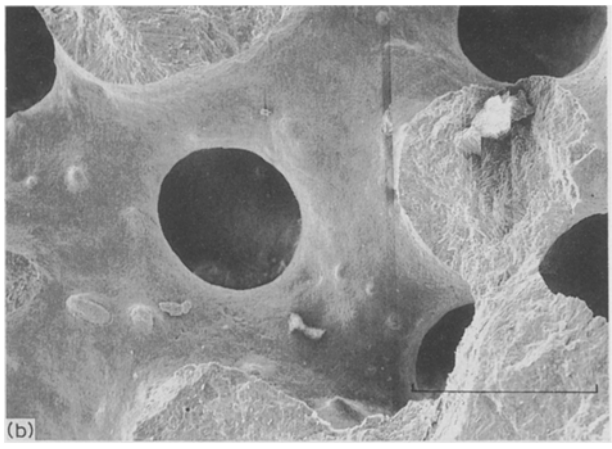
The surface of natural coral (Fig. 7) exhibited a regular microporosity. Round pores were surrounded by a smooth-looking calcified matrix. Fracture areas were clearly evident as rough surfaced zones (Fig. 7a, b). At higher magnification SEM analysis revealed a chiselled interconnected microporosity. On the contrary, the fracture area showed a dense zone, where large crystals appeared to be rigidly locked one with another with no porosity between (Fig. 7c).

Cell-infiltrated coral specimens after 15 days (Fig. 8) showed numerous cells settled in delimited areas. They were round in shape and spread in multilayers (Fig. 8a). They had reached confluency and some of them demonstrated tightly joined pseudopodia (Fig. 8b). At higher magnifications, blebs on the surface of the cells gave them an appearance of a rough-looking membrane. Presence of collagen was not noted. Large cytoplasmic extensions were observed extending along the coral microporosity (Fig. 8c).

5. Discussion

The MTT assay is a rapid, inexpensive, reproducible and semi-automated colourimetric test. To demonstrate the influence of incubation time, Finlay *et al.* [11] cultured cells from four different human leukemia cell lines. Cell growth was counted by means of the colourimetric method of Mosmann, in which the time





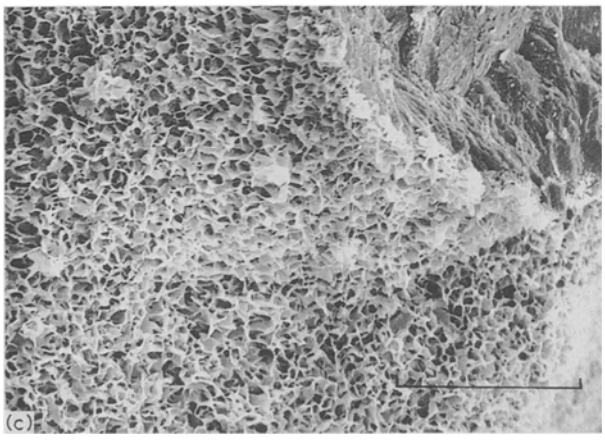


Figure 7 Natural coral surface: (a) scale bar = 750 μm; (b) scale bar = 136 μm; (c) scale bar = 12 μm.

of incubation varied from 0.5 to 5 h. It has been demonstrated that the rate of colour development is dependent on the cell line, time of incubation and perhaps on cell densities. In relation to these results and the cell density, the present experiment used a 3 h incubation time.

The curve of optical density values against corresponding manual countings (Fig. 5) was linear, but did not pass through the origin. This may be due to the use of isopropanol as a solvent, in that Carmichaël *et al.* [8] observed better absorption characteristics when using DMSO as a solvent. A study is currently underway in this laboratory comparing isopropanol to DMSO. Results will determine which solvent will be used in the future to study cell growth on coral.

Additional modifications to the original protocol resulted in an increase of cellular growth. Crushing of the coral specimens should be routinely used in order to improve the accuracy of cell quantification with a colourimetric method. The organization of the porosity did not allow isopropanol to reach and rapidly dissolve the formazan salts synthesized by the cells in the deepest pores. The systematic underestimation found in this experiment when samples remained intact was about 10% and could vary more depending on the biomaterial and porosity.

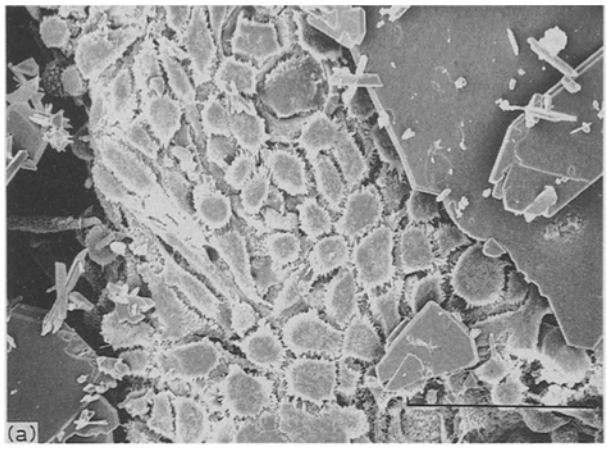
Human fibroblasts cultured on coral surfaces without pre-infiltrated medium exhibited at day 6 a maximum cell growth which represented only 42% of the maximal growth on plastic membrane under the same conditions. Pre-infiltration of coral specimens with medium supplemented with 10% FCS allowed an extension of the growing period by 2 days and brought the number of cells grown into the coral up to 49% compared to cells grown on plastic tubes. This increase did not result in a drastic change in cell growth. It is suggested that at the time of seeding a number of cells invaded the pores and then were not fed by fresh medium. For this reason later changes of media were done by drip-feeding.

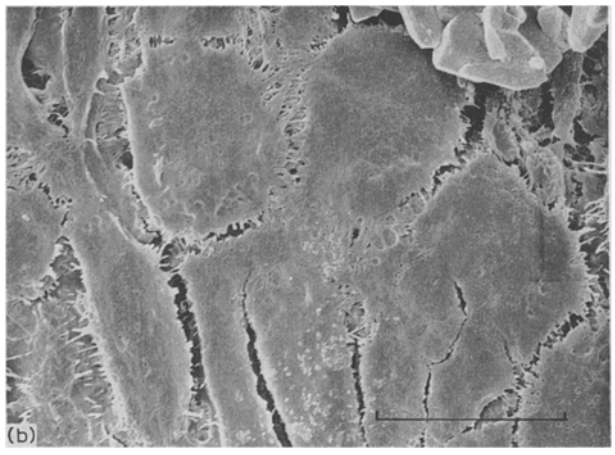
5.1. Final modification and analysis

The use of glass holders avoided the possibility of cell migration from coral to treated plastic. The combination of glass holders, medium pre-infiltrated coral specimens and drip-feeding allowed an extension of the growing period by 9 days, resulting in an increase in fibroblastic population of 1770% over the initial population seeded.

The results of Section 4 demonstrated that at the early time periods the growth of fibroblasts was significantly lower and slower on and into coral specimens when compared to the treated plastic. Several hypothesis can explain this feature.

1. Plastic has been shown to represent a better adhesion support than materials tested for orthopaedic purposes, particularly synthetic mineral materials. Matsuda *et al.* [12] studied fibroblastic growth on bioglass and silica glass and suggested that bioglass may have an inhibitory effect on fibroblastic growth. Similarly, Puleo and colleagues, using osteoblast cultures on a variety of orthopaedic implant materials [13], showed that osteoblasts attached more rapidly on tissue culture polystyrene than on hydroxyapatite or borosilicate glasses. They also showed that the doubling time was significantly lower for polysterene when compared to hydroxyapatite.
2. SEM studies have shown different types of surfaces on the same coral specimen. Some appeared smooth while others appeared rough. It is not known at this time which is preferable for fibroblastic attachment.
3. The low cell proliferation during the early time periods could be caused by a poor seeding cell density





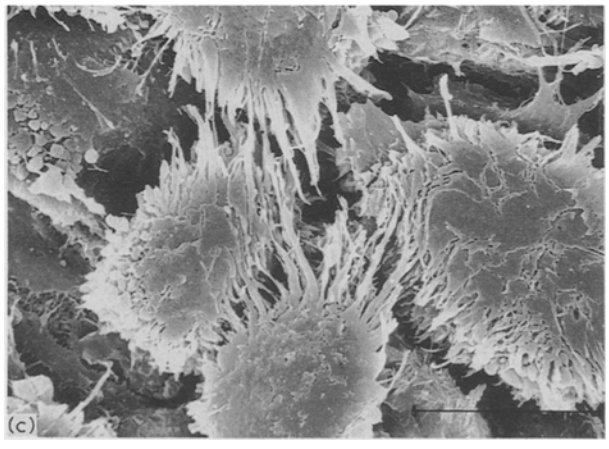


Figure 8 Natural coral specimen pre-infiltrated, fibroblasts having been cultured for 14 days: (a) scale bar = 60 μm; (b) scale bar = 10 μm; (c) scale bar = 8.6 μm.

with regard to the large surface (50% porosity volume of the coralline species).

While a larger surface area may be detremental to early cell proliferation, it became an advantage and allowed a better cell development at later times. This was shown in results on the plastic membranes of the inserts used as controls, which developed a 734% increase of population when compared to the initial population. Coral skeleton with 50% porosity volume, on the other hand, allowed a 1700% proliferation of the seeded fibroblasts after 18 days in culture. This was a 2.2-fold increase when compared to the plateau value of the controls. The difference may be due to the different thicknesses: 11 μm for controls and 3 mm for coral specimens. The porosity and thickness of the coral specimens results in a huge potential adhesion area. Surface evaluation and quantification of surface area of such porous materials would be of interest to quantitate the cell density, but while this technique is possible it remains difficult and experimental.

Coral is a structurally anisotropic material and specimens are generally machined in a random orientation. For this reason, the available open porosity at the surface of each specimen may vary and result in different levels of cell and nutrient infiltration into the deepest pores. This could also explain some high standard deviation values observed. Cell growth could be maximized by using a culture with a permanent flow of nutrients.

SEM studies demonstrated a lack of collagen matrix secretion. This is in accordance with observations made by Sautier *et al.* [14] at an ultrastructural level of osteoblasts cultured on coral granules. This phenomenon might be due to the lack of ascorbic acid during this experiment. This vitamin is known to enhance collagen formation and secretion in fibroblasts [15]. The presence of blebs suggests either synthesis of collagen or hydroxyapatite crystals. Further investigations are necessary to identify the contents of the blebs.

Conclusion

The aim of the experiment was to determine if natural coral skeleton offered a suitable surface for cell growth and if the large area due to the porous architecture allowed a subsequent increase in cell development. While numerous modifications in cell culture and evaluation procedures were required, an efficacious method was resolved.

Results showed that fibroblasts grew on and into the porous natural coral. A 2.2-fold increase in the number of cells was noted in the porous coral specimens when compared to plastic insert controls.

Questions remain as to whether similar behaviour will be seen with thicker specimens and whether there is a maximal thickness for cell viability within the deepest regions of coral.

Coral promises to be an interesting surface for cell growth and adhesion *in vitro*, as well as useful resorbable bone graft substitute *in vivo*.

Acknowledgements

The authors thank the Institute of Medical and Dental Bioengineering, Liverpool, where cell culture techniques were taught, Dr C. Damien for editorial assistance and Dr H. Petite for technical assistance with scanning electron microscopy. This work was financially supported by CNRS. Coral specimens (Biocoral®) were provided by Inoteb France.

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Received 15 October 1992
and accepted 2 February 1993