Tridimensional Response of human Dental Follicular Stem Cells onto a Synthetic Hydroxyapatite Scaffold

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In the last decade, extracorporeal bone tissue engineering has found more clinical applications due to the progress and new achievements in the isolation and characterization of stem cells from different sources, as well as, in controlling proliferation and differentiation *in vitro*. The aim of this study is to evaluate the *in vitro* behaviour, morphological structure and extracellular matrix synthesis of human dental follicle stem cells (hDFSCs) isolated from human dental bud, when seeded onto a synthetic hydroxyapatite (HA) scaffold (ENGIpore©). Populations of CD29+, CD90+, CD146+ and CD166+ were sorted by FAC sorter (FACS) analysis and were cultured in osteogenic medium and then, onto the scaffold. These cells were analyzed by optical and electronic microscopy, at week 1 and 6, before and after the differentiation. Light microscopy showed an intense attachment and colonization of the HA scaffold by polygonal-shaped cells. Scanning electron microscopy after six weeks revealed a tri-dimensional organization of the cells and the presence of dense material around the cell clusters. hDFSCs showed participation in protein biosynthesis and demonstrated high proliferation on the synthetic HA scaffold.

Key words —— stem cell, human dental follicle stem cell, bone tissue engineering, synthetic hydroxyapatite

INTRODUCTION

Tissue engineering may provide an alternative to organ and tissue transplantation,[1–5] producing tissue replacements that can restore the structural features and physiological functions of natural tissues *in vivo*. Bone regeneration by autologous cell transplantation in combination with a biodegradable scaffold is one of the most promising techniques being developed in craniofacial surgery.[6]

Synthetic extracellular matrices, derived from biocompatible and biodegradable polymers, provide a temporary scaffolding to guide cell growth and organization of the new-formed tissue. They may also supply specific signals intended to retain tissue-specific gene expression.[7] Hydroxyapatite (HA) provides a natural 3D scaffold with organic fibrous material in bone.[8]

Osteoblasts synthesize and secrete bone collagen fibers and amorphous organic matrix being essential for osteogenesis. They are also involved in the mineralization process exerting a key role in bone regeneration techniques.[9–11]

Osteoblasts can be obtained by isolating and culturing undifferentiated mesenchymal stem cells from embriogenic[12–14] or post-natal tissues.[15] The first are considered to be pluripotent cells,[16, 17] able to differentiate, *in vivo* and *in vitro*. Post-natal stem cells have been isolated from various tissues, including bone marrow,[18] skeletal muscle,[19] trabecular bone,[20] adipose tissue,[21] blood,[22] neural tissue, skin[23–25] and retina.

Undifferentiated cell populations are also present in dental pulp, both in exfoliated deciduous,[26] permanent teeth,[27] and dental papilla.[28] In particular, third molars, because of their incomplete development are commonly associated with immature tissues, the tooth-germ.

Germs consist of the enamel organ, the dental

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papilla and the dental follicle. The dental follicle is a loose ectomesenchymal tissue deriving from cranial neural crest.[29] It is known that dental follicle stem cells (DFSCs) are responsible for the production of root cementum, periodontal ligament (PDL) and alveolar bone.[30] DFSCs contain a heterogeneous cell population: one line was high in proliferative activity but did not display any mineralization behavior, another exhibited high alkaline phosphatase (ALP) activity, indicative of a highly undifferentiated state and a third line endowed with the mineralization characteristics of a same stage alveolar bone line.[31] In fact, DFSCs could be an interesting source for undifferentiated cells able to take up a mesenchymal pathway and would be the model of choice to test for the biocompatibility of several restorative dental materials. Special tissue requirements for growth need to be determined.

Nevertheless, bone tissue engineering is now developing strategies for repair and reconstruction of damaged or lost tissue in the maxillofacial region. Many research trials have been reported to induce tissue regeneration with scaffolds and culturing.[32, 33]

Tissue engineering approaches attempts to create tissue replacement by culturing autologous cells onto three-dimensional matrixes that facilitate progenitor cell migration, proliferation, and differentiation.[34]

In this study, human dental follicle stem cells (hDFSCs) were first characterized and investigated for their capability to differentiate, under appropriate culture conditions, into cells able to secrete an extracellular matrix undergoing mineralization; then a synthetic HA scaffold (ENGIpore^{TM}, Finceramica Faenza, Italy) was added to these cells in culture and the effects evaluated by electronic microscopic analysis (scanning electron microscope; SEM).

MATERIALS AND METHODS

Six healthy patients, aged 12–24 years, who had their third molars extracted, were recruited at the Oral Surgery clinic, University "G. d'Annunzio", Chieti, Italy for this study with informed consent. Six impacted third molars were surgically removed and attached dental follicles were delicately separated from mineralized tissues, immediately after tooth extraction. Then, they were immersed in a digestive solution, consisting of 4 mg/ml dispase I Roche (Basel, Switzerland), 3 mg/ml collagenase Sigma (St. Louis, MO. USA), 1% penicillin/streptomycin (Invitrogen), 1% claritromycin in 1.25 ml of Minimum Essential Medium Eagle, alpha Modification (MEM-alpha) by Sigma-Aldrich, for 1 hr at 37°C, to separate cells; after enzymatic digestion, cells were passed through a 70 μm strainer BD Falcon (Franklin Lakes, NJ USA) and the single-cell suspensions were seeded into plates with 1 ml of MEM-alpha culture medium, and the addition of 20% Fetal Bovine Serum (FBS, Invitrogen), 100 μM 2-phospho-L-ascorbic acid trisodium salt (Sigma), 1% penicillin/streptomycin (Invitrogen) and 1% L-glutamine. Then cells were centrifuged at 4000 × *g* for 10 min, and the pellets obtained were suspended again in the same culture medium, placed in T25 flasks and incubated at 37°C in 5% CO_2. The medium was renewed every three days to remove cells debris. Approximately in three weeks, the adherent cells reached confluence; they were detached by a 10 min treatment at 37°C with Phosphate Buffered Saline (PBS) 0.02%/EDTA and then reseeded onto T75 flasks.

Enzyme Assay —— Levels of ALP in the medium containing dental follicle cells were assayed by monitoring the rate of hydrolysis of *p*-nitrophenylphosphate (pNPP). An aliquot (40 μl) of the medium from each dental follicle cell culture or standard *p*-nitrophenol was transferred to a well of a 96-well plate containing 50 μl/well of assay buffer (0.5 mol/l Na-acetate buffer, pH 9.8, and 0.05 mmol/l $MgCl_2$) and 50 μl/well of warmed 10 mM pNPP was added to each well. Hydrolysis of pNPP was monitored as change in absorbance at 405 nm in a spectrometer Hewlett Packard (Palo Alto, CA USA). All determinations were carried out in triplicate. ALP activity was negative for the used passages, suggesting that the cell stability was high.

For immunophenotypic analysis, about 1.5×10^6 first-passaged cells were harvested, washed three times with PBS, suspended in 0.7 ml PBS and then incubated with 10 μl FITC or PE-labeled monoclonal antibodies against CD14, CD15, CD29, CD34, CD45, CD90, CD146, CD166 and 2 ml fixative solution for 20 min at room temperature. Cells were centrifuged at 300 × *g* for 10 min, put in 1 ml PBS and then analyzed by using a FACSVantage flow cytometer and the CellQuest Software (Becton Dickinson). The calibration was performed with four colors using the Calibrite3 plus APC BD as Becton Dickinson (Franklin Lakes, NJ USA) and analyzed by FACScomp software. Positive cells for

CD29, CD90, CD146 and CD166 were sorted and collected. Some of the sorted cells were analyzed by SEM before they reached confluence, while the other was cultured in complete medium. Non-sorted cells were used as controls.

For SEM analysis, the collected cells were fixed with 3% glutaraldehyde in PBS 0.15 M (pH = 7.4) for 30 min and then washed in PBS 0.15 M for 15 min. To preserve the lipid structures, specimens were gently washed for three changes every 20 min, post-fixed in 2% phosphate-buffered osmium with the addition of saccharose 0.15 M at room temperature for 5 hr, then given two quick changes of distilled water and gradually dehydrated in increasing concentrations of propylene oxide (from 50 to 100%, 10% steps). They were soaked with amyl acetate, carried through critical point drying, according to standard procedure using liquid carbon dioxide, and sputtered with gold-palladium coating. The probes were examined via SEM LEO 435 VP at about 20 kV, high vacuum mode. Images were digitized. Second-passage cells were then collected and a part of them seeded in osteogenic medium at a density of no less than 2×10^7 per ml, while the others were seeded in osteogenic medium with the addition of synthetic porous HA granules (200 mg/culture dish) onto 10 mm dishes. Osteogenic medium was obtained adding to MEM–alpha culture medium 15% FBS (Invitrogen), 1% L-glutamine, 50 μM 2-phospho-L-ascorbic acid trisodium salt (Sigma), 3 mM glycerol-2-phosphate disodium salt (Sigma) and 10 nM dexamethasone, as described by Owen;[32] cells were cultured in these conditions and under a controlled atmosphere (5% CO_2, 95% air, at 37°C) for 6 weeks.

SEM analyses were performed on differentiated cells at week 1 (before the confluence) and at week 6 (after confluence); one group with and the other without the porous HA granules. For light microscope analysis, specimens were fixed for 4 hr with 4% formaldehyde with 2% saccharose in PBS 0.15 M (pH = 7.4), embedded in London Resin (LR) white Taab using TT System (TMA2 Grottammare, Ascoli Picero, Italy). After polymerization, thin sections were prepared using a sawing and grinding technique. The sections were stained with toluidine blue and observed under an Axiolab microscope (ZEISS, Oberkochen, Germany) connected to a digital camera Fuji FinePixS2Pro (FUJIFILM Corporation, Tokyo, Japan). The images were stored in RAF format with 3032×2035 Grid of Pixels.

RESULTS

By day one, and during the first week of culture, on microscopic analysis cells were aggregated in groups with the presence of some cellular debris. At day seven, cells from the initial aggregates started to expand exhibiting a high proliferation rate and assuming a fibroblast-like morphology.

At day fifteen, cells were about 80% confluent (Fig. 1). Immunophenotypic analysis (Fig. 2A), performed by FACsorter (FACS), allowed to discriminate a homogeneous population of cells negative for hematopoietic markers: CD14 (monocyte; macrophage), CD15 (neutrophils; eosinophils; monocyte), CD34 (hematopoietic stem; progenitor cells; endothelium, Fig. 2B) and CD45 (common leukocyte antigen, Fig. 2C), but positive for cell surface antigens exhibited by mesenchymal stem cells (CD29, Fig. 2D), CD90, CD146 and CD166/MUC-18 (Fig. 2E). Table 1 summarizes the analysis results by the FACS, for the hDFSCs. Among the dental follicles obtained from different donors the pattern of marker expression did not vary significantly.

Light Microscopy

We observed an intense attachment and colonization of the HA scaffold by the DFSCs. These cells appeared interconnected with each other and arranged in a reticular way (Fig. 3A). It was possible to distinguish mainly two cell morphologies: one appeared elongated and spindle-shaped with a centrally located nucleus and the other one exhibited a star shaped and cuboidal appearance (Fig. 3B–3C). The active osteoblasts were determined by their relatively large cuboidal shape. The large

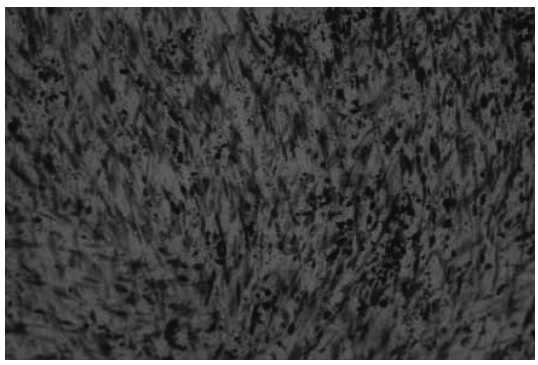

Fig. 1. At Week 2 Cells Showed 80% Confluence

A star-shaped and a spingle like cell populations can be discriminated. Brown granules are discemible in high confluence areas.

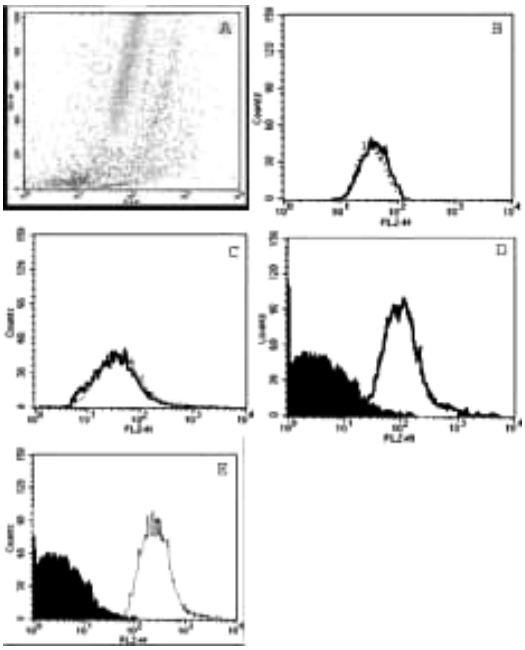

Fig. 2. FACS of hDFSCs at Week 1

(A) Phenotypical characterization of hDFSCs. (B) hDFSCs negative for CD34. (C) hDFSCs negative for CD45. (D) hDFSCs positive for CD29. (E) hDFSCs positive for CD166.

Table 1. FACS Analysis Results for hDFSCs

MUC-18	Evaluation
CD14	—
CD15	—
CD34	—
CD45	—
CD29	++
CD90	++
CD146	++
CD166	++

cuboidal cells were also seen on the HA surface with an adjacent fibroblastic spindle shaped cell lining (Fig. 3D). Cells also colonized the inner surface of the scaffold one through the interconnection pore. We confirm that the cuboidal cells are tightly attached along the entire surface of the pore (Fig. 3E).

Scanning Electron Microscopy

SEM analysis of primary culture cells showed that they have a mesenchymal stem celllike morphologic structure, spindle shaped, with rounded nucleus (diameter about 25 μm), large nucleoli and well defined vacuoles. At higher magnification, cells showed a well organized cytoskeleton, and a large number of typical vesicles, suggesting a high metabolic activity and the participation of these cells in protein biosynthesis and their exocytosis mechanism. When these cells reach confluence they show several cytoplasmatic projections of different length and long filopodia that interconnect with each other (Fig. 4A). Qualitative SEM analysis indicates earlier attachment, more significant cell-cell adhesion, and proliferation. Polygonal mesenchymal stem cells and cell clumps are observed on the HA scaffold with very intimate contact with the micro rough surface of the biomaterial (Fig. 4B).

Osteoprogenitor cells cultured on a HA scaffold reveal a net-like arrangement of cell clusters. The size of clusters increases gradually with culture time.

Moreover, a SEM photograph of osteoprogenitor cells cultured on the scaffold revealed dense material around the cell clusters. It was observed that most of the osteogenic cells were attached to the micro rough surface, but some were observed to be partially penetrating into the scaffold. Sporadic calcified deposits were detected between the granules and on the cell surfaces (Fig. 4C). They seem to be the initial centre of matrix aggregation.

DISCUSSION

Porous HA scaffolds are promising materials for tissue engineering because they offer a tridimensional support and serve as a template for cell proliferation and at last tissue formation.[36–39] Several studies demonstrated that the composition of ceramics influences cell differentiation, since synthesis of bone-specific proteins such as ALP and osteocalcin synthesis are higher on synthetic HA than on brushite.[40] Osteoblasts cultured on pure HA, pure Tricalciumphosphate (TCP), or HA/TCP 70/30 or HA/TCP 35/65 ceramics showed a different type I collagen and ALP mRNA expression.[41]

In our study, the FACS analysis results showed the typical immunophenotypic characterization of mesenchymal cells and SEM detected a high cell proliferation. hDFSCs were positive for CD29, CD90, CD146, CD166 and negative for the markers CD34 and CD45. The cytofluorimetric analysis excludes any presence of hematopoietic progenitors cells in the primary undifferentiated culture. The negative hematopoietic marker CD34, was in accordance with previous findings[10] for culture of cells derived from human periodontal ligament. Furthermore, our cytofluorimetric analysis confirmed that the cells were positive for the mesenchymal marker

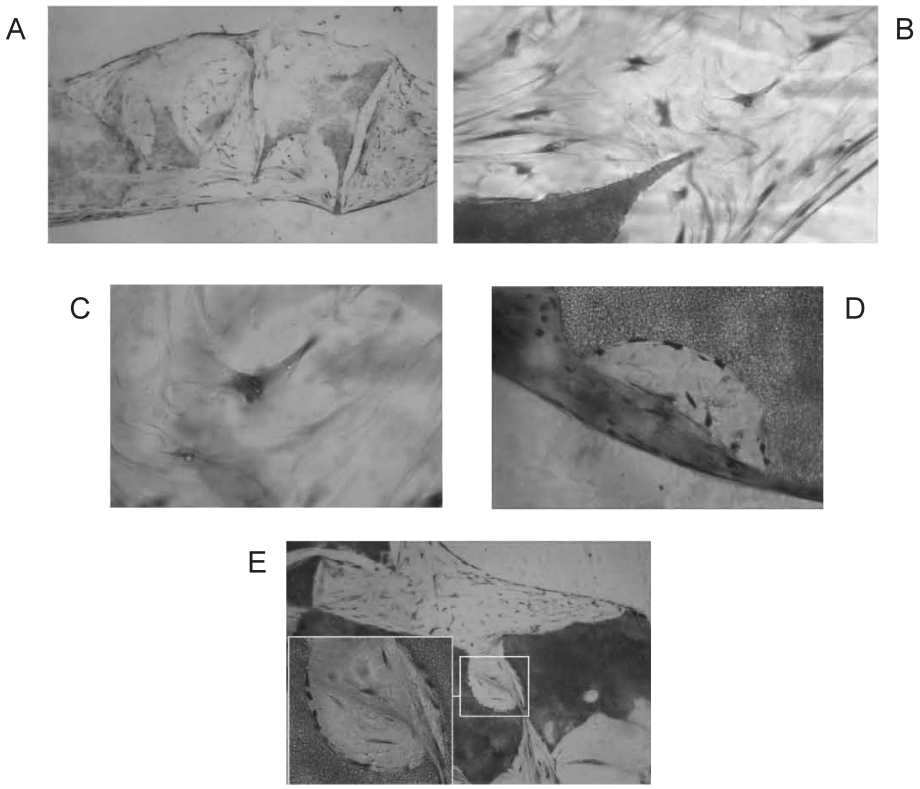

Fig. 3. Colonization of the Hydroxyapatite Scaffold by the Dental Follicle Stem Cells as Seen by Light Microscopy

(A) Colonization of the HA scaffold by the DFSCs. (B) Spindle shapedand star shaped cells with a well defined nucleous and filopodia were discemble. (C) At higher magnification was possible to evaluate the cell morphology of a star shaped cell and the projections of the plasmalemma. (D) Large cuboidal cells on the HA surface with an adjacent spindle shaped fibroblast cell lining. (E) Cells colonizing the inner surface of the scaffold through the interconnection pore. Cuboidal cells appear tightly attached along the whole surface of the pore (selection).

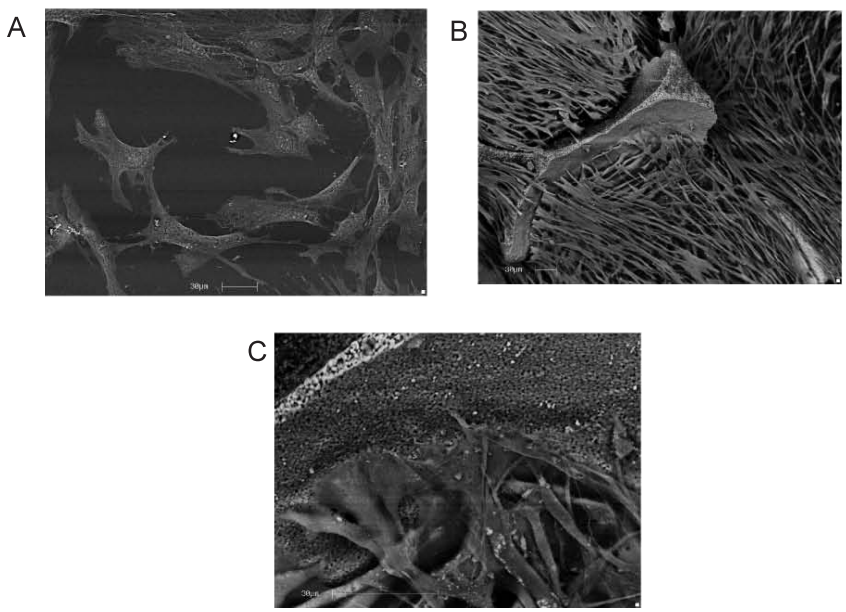

Fig. 4. Dental Follicle Stem Cells Cultured on a HA Scaffold as Seen by SEM

(A) SEM analysis of primary culture cells showed a mesenchymal stem cell-like morphostructure, star-shaped with a well organized cytosckeleton. (B) Colonization of the HA scaffold with a very intimate contact between the micro-rough surface of the biomaterial and the cells. (C) At higher magnification revealed a net-like arrangement and a deposition of granules on the plasmalemma.

CD166 and CD29.

SEM analysis of primary culture cells showed a mesenchymal stem cell-like structure, spindle shaped, similar to the mesenchymal stem cell cultures derived from adult bone marrow.[35, 42, 43] In this study, before confluence, it was possible to define the direction of cell locomotion for the presence of retraction fibers at the back and filopodia and lamellopodia in front. When these cells reached confluence, they showed several cytoplasmatic projections of different length and long filopodia that interconnected the cells with each other. The scaffold architecture allowed the cells, not only to attach to the HA surface, but also to partially penetrate the scaffold. The microporosity may considerably increase the protein absorption capacity of HA, as previously demonstrated.[44] The presence of microporosity inside a macroporous bone substitute favors a change in the cell cytoplasmic extensions and cell spreading but is not absolutely necessary, since cell growth is also effective on macroporous ceramics with only 2% microporosity.[45]

Although, some authors consider that the microporosity has no evident effect on cellular morphology,[46, 47] others, as well as this study, have shown that it plays a role in initial cellular anchorage and attachment,[48] certainly because of the higher adsorption of proteins involved in cell attachment such as fibronectin.

In this *in vitro* experiment, the presence of numerous cytoplasmic organelles suggests the participation of hDFSCs in protein biosynthesis. The HA scaffold supported cellular differentiation and the formed extracellular matrix was filled with mineralized nodules. In fact, HA affected cell attachment and provided the right environment for hDFSCs growth.

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