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In vitro study of adherent mandibular osteoblast-like cells on carrier materials

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Abstract. Augmentation of the craniofacial region is necessary for many aesthetic and reconstructive procedures. Tissue engineering offers a new option to supplement existing treatment regimens. In this procedure, materials composed of hydroxyapatite (HA), of synthetic or natural origin, are used as scaffolds. The aim of this study was to evaluate the effects of three HA materials on cultured human osteoblasts *in vitro*.

Explant cultures of cells from human alveolar bone were established. Human osteoblasts were cultured on the surface of HA calcified from red algae (C GRAFT™/Algipore®), deproteinized bovine HA (Bio-Oss®) and bovine HA carrying the cell binding peptide P-15 (Pep Gen P-15™). Cultured cells were evaluated with respect to cell attachment, proliferation and differentiation. Cells were cultured for 6 and 21 days under osteogenic differentiation conditions, and tissue-culture polystyrene dishes were used as control. The ability of cells to proliferate and form extracellular matrix on these scaffolds was assessed by a DNA quantification assay, protein synthesis analysis and by scanning electron microscopical examination. Osteogenic differentiation was screened by the expression of alkaline phosphatase. The osteoblastic phenotype of the cells was monitored using mRNA levels of the bone-related proteins including osteocalcin, osteopontin and collagen Type I.

We found that cells cultured on C GRAFT™/Algipore® and Pep Gen P-15™ showed a continuous increase in DNA content and protein synthesis. Cells cultured on Bio-Oss® showed a decrease in DNA content from Day 6 ($P < 0.05$) to Day 21 ($P < 0.0001$) and protein synthesis on Day 21 ($P < 0.005$). Alkaline phosphatase activity increased in cells grown on C GRAFT™/Algipore® and Pep Gen P-15™ in contrast to cells grown on Bio-Oss®, in which the lowest levels of activity could be observed on Day 21 ($P < 0.05$). Reverse transcriptase polymerase chain reaction analysis confirmed the osteoblastic phenotype of the cells grown on all three materials throughout the whole culture period.

The results of our *in vitro* study show that the differences in metabolic activity of cells grown on HA materials are directly related to the substrate on which they are grown. They confirm the excellent properties of HA carrying the cell binding peptide P-15 and HA calcified from red algae as used in maxillofacial surgery procedures.

Key words: bone tissue engineering; hydroxyapatite; differentiation; human osteoblasts; proliferation; RT-PCR.

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Augmentation of the craniofacial region is necessary during numerous aesthetic and reconstructive procedures. Autogenous grafts – such as cortical and cancellous bone, cartilage and periosteum; banked or processed bone grafts; free bone flaps; allogenous grafts; and alloplastic materials – have been advocated for this purpose. Autogenous bone grafts are still considered to be the gold standard due to the lack of immunological rejection mechanisms, presence of stem cells and growth factors, and finally their osteoinductive and osteoconductive properties[24,9,16]. However, alternatives to autogenous bone, especially in augmentation of the maxillary sinus for placement of dental implants, have been explored[14], and a clinical trial, postulating that bone augmentation procedures of the maxilla based on tissue-engineering concepts offer significant advantages compared with conventional allografts and autografts, has been carried out[20].

In general, tissue-engineering research has been driven by the challenge to produce tissue replacements that can restore the structural features and physiological functions of natural tissues *in vivo*[13]. Bone generation by autogenous cell transplantation in combination with a biodegradable scaffold is one of the most promising techniques being developed in craniofacial surgery[15]. Calcium phosphate bioceramics, especially hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$), are attractive materials for use in tissue engineering due to their biological properties, such as bioactivity and biodegradability[25,27]. HA is commonly used in maxillofacial surgery, especially for sinus floor augmentation, because HA shows excellent tissue response and good osteoconductivity[14,18].

Although detailed histological comparisons using experimental human or animal models *in vivo* are important, it is difficult to evaluate the materials with respect to the degree of osteoconductivity. *In vitro* comparison of materials using human osteoblast-like cells could elicit more consistent and clear results than *in vivo* comparison.

In this study, granules of different origin were compared: natural HA calcified from red algae (C GRAFT™/Algipore®), deproteinized natural bovine HA (Bio-Oss®) and anorganic bovine HA carrying the cell binding peptide P-15 (Pep Gen P-15™). C GRAFT™/Algipore® (obtained from The CLINICIAN'S PREFERENCE LLC, Golden, CO, USA; particle size, 0.5–1.0 mm), with its well-known mechanical and physico-chemical properties, has proved to have the biocompatible properties necessary for a bone graft substitute *in vivo*[4,5,21]. Pep Gen P-15™ (obtained from DENTSPLY Friadent Ceramed, Lakewood, USA; particle size, 0.25–0.42 mm), with a synthetic cell binding peptide (P-15: potent cell binding domain in the alpha 1 (I) chain of bone collagen Type I[2,19]), provides an environment that is permissive for cell migration, cell differentiation and morphogenesis[7]. Finally, Bio-Oss® (obtained from Geistlich-Pharma, Wolhunsen, Switzerland; particle size, 0.25–1.0 mm) has been widely utilized in basic and clinical dentistry as a material for sinus floor augmentation[26], and is a possible choice for bone grafting by its promotion of osteoblastic activity in the early stages of bone regeneration[22,1].

In our study, we evaluated the initial cell attachment, proliferation and differentiation, and matrix mineralization of human osteoblasts on the surface of these HA granules, using primary osteoblast-like cells and tissue-culture polystyrene dishes (TCPS) as control. The HA granules were cultivated for 6 and 21 days under osteogenic differentiation conditions.

Materials and methods

Cells and cell culture conditions

For isolation of osteoblast-like cells, a specimen of cortico-cancellous bone of the mandible was obtained from a healthy individual. The biopsy was washed extensively in phosphate-buffered saline (PBS; Gibco Life Technologies, Grand Island, NY, USA); the attached soft tissue and periosteum were removed. Bone was cut into pieces of approximately 2 mm × 2 mm and seeded onto 25-cm^2 polystyrene flasks in 3 ml of standard culture medium consisting of DMEM/Ham's F-12 1:1 (Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum (FCS; Gold, PAA Laboratories, Cölbe, Germany), 1% 200 mM L-glutamine, 1% HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco Life Technologies, Grand Island, NY, USA). Medium was changed every 48 h and was used for osteoblastic cells in primary culture until subculture. Cells were cultured until confluence in a controlled atmosphere (5% CO_2, 95% air, and 37 °C). The bone fragments were removed after 2 weeks and cells were passaged with trypsin–EDTA solution (PAA Laboratories), counted on CASY 1® (Schärfe System GmbH, Reutlingen, Germany) and plated in 75-cm^2 culture flasks. For further investigation cells of the second, third and fourth passage were collected and seeded at a density of 4.10^5 cells/culture dish in 10-mm TCPS. The HA granules were added (200 mg/culture dish) to the wells and cultured with osteogenic culture medium which consists of DMEM/Ham's F-12 1:1 (Biochrom) supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, 0.28 mM ascorbic acid and 230 mg/l $CaCl_2$ (all from Sigma, St. Louis, MO, USA). Additionally, the same amount of cells were seeded in TCPS without HA granules as a control. The cells were maintained at 37 °C in a fully humified atmosphere at 5% CO_2 in air. The culture medium was changed two or three times per week.

SEM imaging of cells on HA granule surfaces

For SEM, the HA granules were washed twice with PBS and fixed in 2.5% glutaraldehyde in phosphate buffer pH 7.5, rinsed three times with PBS and dehydrated in a graded ethanol series. The samples were critical-point dried and gold sputtered, and then examined with a Joel 6310 scanning electron microscope (Joel, Tokyo, Japan).

Cell harvesting and DNA assay

After 6 and 21 days cells were collected from the dishes using a 1 mg/ml papain solution in standard culture medium (Sigma, St. Louis, MO, USA) modified from a procedure published by Young & Levinson[28]. Dishes were covered with 3 ml papain solution and incubated for 5 min at 37 °C. Detached cells were collected and another 3 ml of papain solution were added followed by incubation under the same conditions. Detached cells were collected and used for the determination of cell number, DNA content and alkaline phosphatase (ALP) activity, and protein and RNA extraction. The amount of DNA was determined using a PicoGreen® dsDNA-Quantification-Kit (Molecular Probes, USA). The cell pellets were homogenized in a lysis solution (0.2% v/v Triton X-100, 10 mM Tris (pH 7.0), 1 mM EDTA) after two cycles of freezing/thawing for 45–60 min at room temperature. The DNA solution obtained was spectrometrically analysed using emissions measured at 485 and 520 nm by a Hitachi U-2000 Spectrophotometer (Hitachi High-Technologies, Krefeld, Germany). The amount of DNA was related to cell number using the standard curve of cell number versus emission measured (nm).

Protein synthesis and alkaline phosphatase specific activity

For protein isolation, cells were incubated in a lysis solution (1% Nonident NP40,