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Using human neural crest-derived progenitor cells to investigate osteogenesis: An *in vitro* study

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ABSTRACT

Human tooth contains a distinct population of neural crest-derived progenitor cells (dNC-PCs) which are known to give rise to specialized daughter cells of an osteogenic lineage. We hypothesised that dNC-PCs could develop into neural crest-derived bone in a self-propagating and extracorporal culture system. Thus, we examined the three-dimensional structure obtained from osteogenic-stimulated dNC-PCs by morphological, biochemical and spectroscopic methods. After the onset of stimulation, cells formed a multilayer with outer cells covering the surface and inner cells secreting a hyaline matrix. With prolonged culture, multilayers contracted and formed a three-dimensional construct which subsequently converted to a calcified mass. Differentiation of progenitor cells was associated with apoptosis. Cell types which survived were smooth muscle actin-positive cells and bone-like cells. The expression of osteoblastic markers and the secretion of a collagenous matrix indicate that the bone cells had acquired their functional phenotype. Furthermore, these cells produced and secreted membrane-bound vesicles into the newly forming matrix. Consequently, an early biomineralized extracellular matrix was found with calcium phosphate deposits being associated with the newly formed collagen matrix framework. The molar calcium–phosphorus-ratio of the mineralized collagen indicated that amorphous calcium phosphate was present within this matrix. The data suggest that stimulated cultures of dNC-PCs are able to recapitulate some processes of the early phase of osteogenesis.

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1. Introduction

A variety of different stem cells have been identified in many animal and human tissues (Young et al., 1999). Adult stem or progenitor cells are being widely investigated with a focus on their ability to differentiate into a broad array of cell types potentially useful as cell material for purposes of tissue engineering and regenerative medicine (Kassem, 2006; Aejaz et al., 2007; Atala, 2007; Redi et al., 2007; Bajada et al., 2008). The developing field of craniofacial tissue engineering promises the regeneration of dental, oral, and craniofacial structures (Mao et al., 2006). Recent evidence suggests that the connective tissue of the human tooth contains a particular population of neural crest-derived progenitor cells (termed dNC-PCs) which give rise to a wide range of specialized daughter cells when outside their dental signal network (Degistirici et al., 2008; Schoenebeck et al., 2009). In attempting to use dNC-PCs for tissue engineering we showed that it was possible to induce bone formation in a nude mouse model by combining dNC-PCs with bovine bone-derived granulates, which act as a scaffolding component. Under these conditions, the only differentiated stem cells observed were those of an osteogenic lineage (Degistirici et al., 2008). To take full advantage of dNC-PCs, however, it will be important to understand how these cells differentiate into bone-specific cell types and what environmental conditions are stimulating these cells to become functional bone.

In craniofacial tissues, the bone cell lineages originate from cranial neural crest cells that have committed to the osteogenic cell lineage becoming osteoprogenitor cells, such as preosteoblasts, osteoblasts, and osteocytes (Le Douarin et al., 1994; Dupin et al., 2006; Chai and Maxson, 2006). It turns out that the molecular mechanisms inducing osteogenesis in cranial neural crest cells, which produce the facial and jaw skeleton, are distinct from those operating in mesodermal cells, which produce most of the skeleton (Helms and Schneider, 2003; Tucker and Lumsden, 2004; Abzhanov et al., 2007; Han et al., 2007; Xu et al., 2007; Deng et al., 2008). Therefore, our focus is on the specific

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biology of the process of dNC-PCs developing into bone-like tissue. Aspects considered are the differentiation of dNC-PCs from stem or progenitor cells to cells of the osteogenic lineage and the cellular changes that accompany their differentiation. Our current notion is that the extracellular matrix components and their structural architecture trigger this differentiation process, thus we investigated the potential of dNC-PCs for osteogenesis and bone development when cultured in osteogenic growth medium and maintained within a self-established extracorporal microenvironment.

Here, we report that dNC-PC cultures responded to osteogenic stimulation by progressing through a series of structural and biochemical changes that culminated in the formation of a simply ordered bone-like matrix. Although we are left with several questions, these data give new insights into the molecular processes of human bone formation by stem or progenitor cells derived from the neural crest.

2. Materials and methods

2.1. Cell isolation and expansion culture

Surgically removed impacted third molars of young adults were used to prepare dental neural crest-derived progenitor cells (dNC-PCs) (Degistirici et al., 2008; Schoenebeck et al., 2009). Written consent was obtained from all parents of the participating patients. Briefly, apical pad-like tissue of the developing tooth was cut into small pieces, enzymatically pretreated with collagenase/dispase solution (Sigma, Munich, Germany) and microexplants seeded in tissue culture flasks (BD Falcon, Germany). Subsequently, microexplants were maintained in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) (Cambrex Bio Science, Verviers, Belgium) supplemented with 2 mM glutamine and 1% penicillin/streptomycin (Sigma) and 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany). Upon reaching 70–80% confluence, outgrowing cells were detached by trypsinization (trypsin/EDTA) (Sigma) and replated at a density of 5×10^3 cells/cm². The resulting cells were further incubated under standard culture conditions for expansion. For experiments, dNC-PCs were used at passages 3 to 4.

2.2. Osteogenic culture

For osteogenic induction, cultured dNC-PCs were initially seeded at a density of 8×10^3 cells/cm^2 in 24-well plates (BD Falcon, Germany) and cultured in growth medium containing DMEM-LG, 10% FCS and 1% penicillin/streptomycin. When reaching subconfluency, the growth medium was replaced with differentiation medium, DMEM-LG supplemented with 100 nM dexamethasone, 50 µM ascorbic acid-2 phosphate, and 10 mM ß-glycerol phosphate (all from Sigma). The medium was changed twice a week. For a three-dimensional culture, cells were seeded at a density of 5×10^3 cells/cm^2 in 25-cm² culture flasks and incubated in osteogenic medium as described above. Formation of a three-dimensional structure was brought about by prolonged culture. Samples were analyzed at the indicated time points.

2.3. Assay of alkaline phosphatase activity

Alkaline phosphatase activity was determined in cell lysates using p-nitrophenyl phosphate as a substrate (Sigma). The procedure was carried out as recommended by the manufacturer. Briefly, cells growing on 24-well plates were washed with phosphate-buffered saline (PBS) and incubated with 1% Triton X-100 (Sigma). The resulting lysate was then incubated with substrate and the released p-nitrophenol was measured at 405 nm (PerkinElmer, Waltham, MA). Alkaline phosphatase activity was expressed as µM p-nitrophenol/l/min/sample.

2.4. Quantification of calcium

The total calcium of the samples was measured by the o-cresolphthalein complexone method using the commercial Calcium Assay-CA590 kit (Randox Laboratories, Co Antrim, United Kingdom). The procedure was carried out according to the manufacturer's protocol. Briefly, cells growing on 24-well plates were washed with PBS and extracted in 200 µl of 0.5 N hydrochloric acid for 5 min. Samples were then vigorously shaken for 4–16 h at 4 °C. Calcium determination was done in 96-well plates with 10 µl of test solution and 90 µl of substrate solution mixed up with 100 µl of distilled water. The amount of deposited calcium was determined at 570 nm using a spectrofluorometer (PerkinElmer) and was expressed as µg/sample.

2.5. TUNEL assay

In situ DNA fragmentation was established using the terminal desoxyribonucleotide transferase (TdT)-mediated dUTP nick-end-labelling technique (TUNEL) in paraffin-embedded sections. We used the ApoTag™ plus peroxidase in situ apoptosis detection kit (KIT S7101, Intergen, USA). The staining procedures were performed according to the manufacturer's recommendations. Slides of colorectal cancer (CRC) treated in the same way served as positive control. Negative controls were carried out with CRC slides without exposure to TdT enzyme.

2.6. Histology and immunohistochemistry

The cultured cells were harvested, paraffin-embedded and sectioned following standard protocols. Briefly, samples were fixed in 4% formaldehyde in PBS, washed with PBS and dehydrated with ethanol. After dehydration, samples were washed with toluene, infiltrated with molten paraffin and embedded and sectioned. Sections of 5 µm were prepared for a panel of immunohistochemical examinations. Dewaxed and rehydrated sections were incubated with hydrogen peroxide to block endogenous peroxidase, antigen retrieval was performed in a hot water bath. The following monoclonal primary antibodies were used: anti-smooth-muscle actin (dilution 1:500; Dako, Hamburg, Germany), anti-osteocalcin (1:500; Acris-Antibodies, Herford, Germany), and anti-collagen type II (1:500; MP-Biomedicals, Illkirch, France). For primary antibody detection the Zytomed Polymer Kit DAB (dilution 1:10; Transduction Laboratories, San Diego, CA, U.S.A) was used. All immunohistochemical stains were performed with an automated staining device (Dako Autostainer, Glostrup, Denmark). Omission of the primary antibodies served as negative controls. Selected sections were stained with hematoxylin and eosin. To identify the formation of mineralization, monolayers were stained with von Kossa. To detect alkaline phosphatase, monolayers were stained with naphtol and fast red violet.

2.7. Electron microscopy

For transmission electron microscopy, samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h at room temperature, washed in cacodylate buffer, post-fixed with 1% osmium tetroxide in cacodylate buffer, dehydrated with ethanol and propylene oxide and embedded in epoxy resin. Ultrathin sections were mounted on copper grids, double-stained with uranyl acetate and lead citrate and examined with a Zeiss TEM 902A.

For scanning electron microscopy, samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h at room temperature. After repeated washings in cacodylate buffer, samples were dehydrated with ethanol and dried using hexamethyldisilazane (Polysciences, Eppelheim, Germany) as the drying medium. Then samples were sputtered with a conductive layer of gold and imaged

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