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In search of representative models of human bone-forming cells for cytocompatibility studies

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ABSTRACT

Osteosarcoma-derived cells have been routinely used for studying osteoblastic functions, but it remains unclear to what extent they mimic the behavior of primary osteoblasts in the study of cells and materials interactions. This study reports comparatively on the responses of three human osteosarcoma cell lines, MG-63, Saos-2 and U-2 OS, and human primary osteoblasts cultured on Ti6Al4V surfaces or exposed to Ti particles. Phenotypic characterization of the cell lines revealed that Saos-2 cells and primary osteoblasts displayed similar expression patterns of Cbfa1, SP7 and osteocalcin. Unlike primary cells, the cell lines expressed markers of undifferentiated cells, had high proliferative rates and poor fibronectin matrix assembly. None of the three cell lines faithfully reproduced the adhesive behavior of primary osteoblasts when cultured on Ti6Al4V surfaces or exposed to Ti particles. Differences in cell growth between the cell lines and primary osteoblasts cultured on Ti6Al4V surfaces were also observed. Ti particles inhibited the growth of Saos-2 cells and primary osteoblasts to a similar extent, while no such effect was observed in U-2 OS and MG-63 cells. Saos-2 cells reproduced the alkaline phosphatase (ALP) activity profile of primary osteoblasts cultured on metallic surfaces or exposed to particles. Altogether, these results show that none of the osteoblast-like cells studied perfectly mimic the behavior of human osteoblast cells (hOB) on Ti6Al4V surfaces or exposed to Ti particles. Saos-2 cells reproduce some of the hOB responses such as the profile of enzymatic ALP activity when cultured on the surfaces or treated with particles as well as cell growth inhibition when exposed to Ti particles. Although *in vitro* cytocompatibility studies involve the evaluation of multiple parameters, Saos-2 cells may be used as representative of human osteoblasts when these standard tests are evaluated.

1. Introduction

Osteoblasts are the skeletal cells responsible for the new bone formation around orthopedic implants. These cells of mesenchymal origin exhibit the specific ability to synthesize the osteoid constituents required for the process of bone matrix deposition [1]. Many cell lines derived from osteosarcoma tumors display features characteristic of osteoblasts, such as the ability to express specific receptors for 1,25-dihydroxyvitamin D_3 and parathyroid hormone, to organize a cell-matrix layer displaying alkaline phosphatase (ALP) activity or to produce specific bone matrix proteins [2–4]. However, the tumor origin of osteosarcoma cells along with cellular immortality also led to significant phenotypic differences [5,6]. The osteoblast-like cells differ markedly from primary osteoblasts in their morphology, mitotic rate and expression profile of several cytokines, growth factors and matrix proteins, as well as in their mineralization activity [7–10]. Besides aberrant gene regulation, such differences have been related to the maturation status of immortalized cell lines within the osteoblast development lineage.

Biomaterials designed for bone repair must have adequate mechanical properties, long-term dimensional stability, excellent abrasion resistance and good biocompatibility. Metallic biomaterials are widely used for manufacturing orthopedic implants. The best candidates for components with high mechanical strength requirements are titanium (Ti) and its alloys [11]. Ti6Al4V is the most widely used Ti alloy for the fabrication of orthopedic devices, since it features a combination of advantageous bulk mechanical strength, good corrosion resistance and excellent biocompatibility. This alloy is able to osseointegrate, thus allowing osteoblast functions on its surface, such as adhesion, proliferation and secretion of specific matrix proteins. Despite the relatively good corrosion resistance of Ti alloys, functional loading for long service periods leads to the release of metallic particles which contribute to periprosthetic osteolysis and subsequent implant loosening [12–14]. *In vitro* studies on the influence of implantable biomaterials on cell

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behavior are commonly carried out to explore their cytocompatibility. Moreover, understanding the response of bone cells to surfaces and particles is essential for the rational development of novel biomaterials which improve implant stability and durability. Owing to the advantages that immortalized cells offer over their respective primary cell counterparts, such as ease of culture and reproducibility of results, a good number of works have investigated the cytocompatibility of implantable materials using osteosarcoma cell lines in place of primary osteoblasts. However, there is a general lack of systematic studies comparing the responses of osteosarcoma and primary cells to biomaterials, thus bringing into question the utility of cell lines as suitable models for cytocompatibility studies.

This study reports comparatively on the behavior of three human osteosarcoma-derived cell lines, MG-63, Saos-2 and U-2 OS, and human primary osteoblasts when cultured on Ti6Al4V surfaces, the most common metal alloy used in total-joint arthroplasty devices. As a significant amount of metallic debris has been detected in retrieval studies of tissues surrounding failed Ti-based implants [12,13], cell responses were also analyzed upon incubation of the cells with Ti particles. To better understand the cell responses to materials, the phenotypic characteristics of the tested cell types, such as growth kinetics, stem cell attributes, extracellular matrix synthesis and osteoblastic-specific gene expression were also addressed.

2. Materials and methods

2.1. Ti6Al4V discs and Ti particles

Discs 20 mm in diameter and 2 mm thick were removed from an extruded bar of Ti6Al4V ELI alloy (90% Ti, 6% Al 4% V, wt.%) by electrospark erosion. Samples were first abraded by grinding their surface with silicon carbides papers of decreasing grain size to remove the outermost part of the discs, and finally polished with 1-μm diamond paste to get a mirror-like finish. Discs were ultrasonically washed in distilled water, cleaned with isopropanol alcohol and sterilized under UV light in a laminar flow hood. Sterilized samples were stored at least 3 days before use in cell culture experiments.

Commercially pure Ti particles, purchased from Johnson Matthey (Ward Hill, MA, USA) were characterized in the course of previously reported work [15]. The mean equivalent circle diameter sizes of Ti particles were 3.32 ± 2.39 μm (range 1–15 μm, 89% <7 μm). These dimensions can be regarded as representative of those measured in metallic debris generated *in vivo*. Ti particles were weighed and sterilized by incubation in isopropanol at room temperature, and dried under UV light in a laminar flow hood. Immediately prior to their addition to the cells, particles were resuspended in culture medium (20 mg ml^{-1}), sonicated at maximum power for 10 min in a bath sonicator (Bransonic 12, Branson Ultrasonidos SAE, Barcelona, Spain) and thoroughly vortexed to obtain a homogeneous particle suspension.

2.2. Cell culture and treatments

Human osteoblast cells (hOB) were isolated from trabecular bone explants aseptically collected from old donors (age 70–80 years) and cultured using a standardized technique, as previously described [16]. Each bone sample was processed in a separated primary culture, and experiments were performed using independent cultures obtained from 18 different patients. Patients enrolled in this research signed an informed consent, and all procedures using human tissue designated "surgical waste" were approved by the Human Research Committee of Hospital Universitario La Paz (Date of Approval: 05-16-2009). Bone fragments were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) heat-inactivated fetal bovine serum (FBS), 500 UI ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. The osteoblastic phenotype of cells derived from bone explants was routinely confirmed by cytochemical measurement of ALP. To avoid dedifferentiation, primary osteoblasts at passage 1–2 were used for the experiments. The human osteosarcoma cell lines (Saos-2, U-2 OS, MG-63) were purchased from ECACC (Salisbury, Wiltshire, UK) and grown in DMEM containing 10% (v/v) heat-inactivated FBS, 500 UI ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. Cells were maintained at 37 °C in 5% CO_2 in a humidified incubator. Cell lines at passage <20 were used for the experiments. Optimal cell densities for studying specific cell functions were determined during the course of previous studies [16–19].

For Ti particle treatments, particle suspensions at 20 mg ml^{-1} were prepared in growth media and added to cells to achieve doses of 10 or 50 ng $cell^{-1}$. Particles and culture media were endotoxin free, as demonstrated by the Sigma E-TOXATE assay for detection and semi-quantification of endotoxins (Sigma, Madrid, Spain). Particles and culture media used in this study contained levels of endotoxins <0.015 EU ml^{-1}.

2.3. Real-time polymerase chain reaction (PCR)

Total RNA was prepared from 7×10^5 cells cultured on tissue culture-treated polystyrene (TCP) for 1 day using TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH, USA), following the procedure described by the manufacturer. Complementary DNA (cDNA) were prepared from total RNA using AMV (Roche Applied Science, Indianapolis, IN, USA) and random hexamers. Real-time quantitative PCR was performed using LightCycler FastStart DNA Master SYBR Green I and LightCycler detector (both from Roche Applied Science). Assays were conducted in duplicate. Quantitative expression values were extrapolated from standard curves and normalized to β2-microglobulin. Specific oligonucleotide primers used were as follows: core binding factor alpha1 (Cbfa1), 5′-ATGATGACACTGCCACCTCTGA-3′ (forward primer, F), 5′-GGCTGGATAGTGCATTCGTG-3′ (reverse primer, R); Osterix (SP7), 5′-TCCCTGCTTGAGGAGGAAGTT-3′ (F), 5′-GCATCCCCCATGGTTTTG-3′ (R); Osteocalcin (OC), 5′-CCTCACACTCCTCGCCCTATT-3′ (F), 5′-CCTGCTTGGACACAAAGGCT-3′ (R) and β2-microglobulin (B2M), 5′-CCAGCAGAGAATGGAAAGTC-3′ (F), 5′-GATGCTGCTTACATGTCTCG-3′ (R).

2.4. Immunoblot assays

Nuclear extracts from 4×10^6 osteosarcoma cells cultured on TCP for 1 day were prepared as previously described [20]. After 5 min boiling at 99 °C, aliquots of 20 μg nuclear extracts were separated on SDS–polyacrylamide (10% w/v) gels. Proteins were electroblotted to PVDF membrane (Bio-Rad Laboratories, Madrid, Spain) at 100 mA for 2 h using a protein transfer unit (Bio-Rad Laboratories). The membranes were then blocked and incubated in goat anti-Cbfa1 pAb, rabbit anti-SP7 pAb and mouse anti-PCNA mAb (Santa Cruz, Heidelberg, Germany) overnight at 4 °C. Horseradish peroxidase-conjugated rabbit anti-goat or anti-mouse IgG (Dako, Glostrup, Denmark) were used as secondary antibodies for detection. The membranes were developed with an enhanced chemiluminescence western blotting detection kit (Perkin–Elmer Life Sciences, Boston, MA), following the procedure described by the manufacturer.

2.5. Gel retardation assays

Partially complementary oligonucleotides were prepared and, when required, labeled with [α-32P]dCTP, as reported earlier