Human mesenchymal stem cells and biomaterials interaction: a promising synergy to improve spine fusion

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Abstract

Purpose Spine fusion is the gold standard treatment in degenerative and traumatic spine diseases. The bone regenerative medicine needs (i) in vitro functionally active osteoblasts, and/or (ii) the in vivo induction of the tissue. The bone tissue engineering seems to be a very promising approach for the effectiveness of orthopedic surgical procedures, clinical applications are often hampered by the limited availability of bone allograft or substitutes. New biomaterials have been recently developed for the orthopedic applications. The main characteristics of these scaffolds are the ability to induce the bone tissue formation by generating an appropriate environment for (i) the cell growth and (ii) recruiting precursor bone cells for the proliferation and differentiation. A new prototype of biomaterials known as “bioceramics” may own these features. Bioceramics are bone substitutes mainly composed of calcium and phosphate complex salt derivatives.

Methods In this study, the characteristics bioceramics bone substitutes have been tested with human mesenchymal stem cells obtained from the bone marrow of adult orthopedic patients.

Results These cellular models can be employed to characterize in vitro the behavior of different biomaterials,

which are used as bone void fillers or three-dimensional scaffolds.

Conclusions Human mesenchymal stem cells in combination with biomaterials seem to be good alternative to the autologous or allogenic bone fusion in spine surgery. The cellular model used in our study is a useful tool for investigating cytocompatibility and biological features of HA-derived scaffolds.

Keywords Spine fusion · Scaffold · Bone · Stem cell · Proliferation

Introduction

Spine fusion is frequently used to treat traumatic and degenerative spine disease [1]. Autogenous bone graft harvested from the iliac crest has long been the gold standard for fusion procedures due to its osteoinductive and osteoconductive properties. However, there is significant morbidity associated with the harvest procedure [2]. Allograft bone is routinely used as an alternative to autogenous bone but concerns about immunogenicity and disease transmission surround its use [3]. Additionally, allograft bone has been used successfully in the cervical and lumbar spine, but fusion rates for multilevel cervical and posterior lumbar procedures are much lower [4]. These and other difficulties with bone grafts drive intensive research efforts to develop alternative spinal fusion procedures.

The regenerative medicine is becoming available in the clinical practice. Indeed, functionally active human tissues in vitro and their regeneration in vivo have been recently obtained. In the orthopedic application, the availability of bone allograft or substitutes is of a paramount importance

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for the effectiveness of surgical procedures. In the orthopedic research and clinical practice, the bone tissue engineering is an emerging issue. In earlier studies, the bone tissue engineering was addressed to the development of biomaterials to be employed *in vivo* for implantation, void of the rejection effect. More recently, new scaffolds were set up owing the ability (i) to generate a microenvironment which induces the cellular growth; (ii) to recruit, in the surrounding of the implant site, bone precursor cells (osteoconductivity), and (iii) to induce cell proliferation and differentiation for the bone tissue development (osteinductivity).

The reconstruction of large bone segments remains a critical clinical problem in the case of extensive bone loss due to pathological events such as trauma, inflammation, degeneration and surgical treatment of tumors [5, 6]. For this purpose, several approaches have been attempted for defect filling and subsequent regeneration, including autogenous and xenogenous bone grafting and synthetic biomaterials [7]. Due to its ideal biocompatibility and osteogenic properties, autogenous bone taken from a secondary surgical site has been widely utilized and still considered as the “golden standard”, such as the autogenous bone tissue derived from iliac crest grafted onto the intertransverse process for the fusion of the lumbar spine [8, 9]. However, the use of autogenous bone has limitations, including availability and unpredictable healing kinetics. Moreover, donor site pain and potential post-surgical infections are common complications associated with such procedure [9]. These limitations and considerable recent progress in biotechnology have driven the development of synthetic materials/scaffolds engineered specifically for bone replacement applications [7, 9, 10].

Over the last 10 years, attention has been addressed to the development of optimized synthetic or semi-synthetic substitutes for autogenous bone grafting [6]. New calcium- and phosphate-based substitutes have been developed, including the generation of the class of biomaterials named “bioceramics”. These scaffolds include bioactive ceramics which form direct chemical bonds with bone or even with soft tissue of a living organism. Different bioactive scaffolds have been recently tested in animal models and also in clinical trials in order to find bone graft alternatives for vertebral fusion in spine surgery [11, 12]. Moreover, biomaterials are now often combined to bone marrow-derived mesenchymal stem cells in order to enhance the osteoinductive potential of the bone substitutes, with interesting results concerning spinal fusion [13–15].

Thus, the basic research on synthetic biomaterials and their osteogenic properties is of great interest for its clinical implications. To test the interaction between engineered biomaterials and bone cell precursors different cellular models have been used. Biomaterial properties have been

assayed to verify their involvement in differentiation and developmental processes. In recent years, the improvement of the knowledge of the adult stem cells biology and of mesenchymal stem cell features allowed their use to repair adult tissues, bone included. Human mesenchymal stem cells, as a cellular model, are employed to directly assay bone cell precursors and to test the effect elicited on them by new scaffolds developed for bone engineering applications.

Methods

Isolation and growth of human mesenchymal stem cells (hMSCs)

Iliac crest bone marrow aspirates (10 mL) were obtained from orthopedic patients who underwent bone marrow harvesting under general anesthesia. Specimens were obtained according to the tenets of the Declaration of Helsinki and the ethical committee of the “Rizzoli” Orthopedic Institute, Bologna, Italy. All donors provided informed consent for the biopsy. A mononuclear fraction was isolated by Ficoll-mediated (Histopaque, # 10771, Sigma Company, Milan, Italy) discontinuous density-gradient centrifugation and polystyrene adherence capacity. The pellet was suspended and cells were seeded in a cultured with α MEM (Lonza, Milan, Italy) supplemented with 20% fetal bovine serum (FBS), antibiotics and incubated at 37 °C with 5 % CO₂ in a humidified atmosphere. At 2-day intervals, the medium was replaced while non-adherent cells were removed. After 6 days adherent fibroblast-like colonies of hMSCs were clearly visible. Cultures were re-fed weekly and grown to 50–60 % confluence in 2–3 weeks; then hMSCs were trypsinised (0.25 % trypsin and 1 mM EDTA) and expanded in new plastic vessels.

Flow cytometric analysis (FCA) of hMSCs

Cell culture at the first passage was characterized by flow cytometric analysis (FCA) for specific surface antigens, in accordance with the International Society for Cell Therapy. Each sample analyzed by FCA contained 1×10^5 cells. hMSCs were incubated for 30 min at room temperature with specific phycoerythrin (PE) or fluorescein isothiocyanate (FITC) conjugated-antibodies. Positive selection of hMSCs was carried out using the following markers: PE anti-human CD29 (integrin β 1, Biosciences clone IgG1 MEM-101A), PE antimouse/human CD44 (H-CAM, Biosciences clone IgG2b IM7), FITC anti-human CD71 (Biosciences clone IgG1 OKT9) and PE anti-human CD105 (Endoglin, Bioscience clone IgG1 SN6). The negative selection of hMSC was performed with the following

markers: FITC anti-human CD45 (Bioscience, clone IgG1 HI30), PE anti-human CD235a (glycophorin A, Bioscience clone IgG2b HIR2). hMSCs were analyzed using FCA (Becton–Dickinson, Milan, Italy) after PBS washes [16].

Scaffolds and cell loading

Biomaterials named Sintlife and Engipore (Finceramica SpA, Faenza, Italy) were used in the assay with human mesenchymal stem cells (hMSCs). Sintlife is a hydroxyl-apatite scaffolds Mg²⁺-doped and Engipore is a high porosity hydroxyl-apatite scaffolds. ShMSCs at the first passage, with 50–60 % confluence, were detached from the culture flasks and resuspended in culture medium to obtain a cell suspension with a density equal to 1,000 cells/cm² of surface area, in 1 ml, for each scaffold. Tissue culture polystyrene (TCPS) was employed as control. The scaffold was then placed in wells ($\varnothing = 10$ mm) filled with the proper cell suspension and incubated for 2 h. To maximize cell-scaffold interaction, cell suspension was subjected to pipetting every 15 min. After the incubation period, scaffolds were placed in empty wells. Fresh culture medium, 1 ml, was added to each scaffold.

Cell viability assay

Human mesenchymal stem cells were loaded onto HA scaffolds and onto TCPS which was used as control, in 24-well culture plates ($\varnothing = 10$ mm) and cultured as described above. In order to mimic the clinical application environment, the same volume of bone void filler was used for each type of scaffold. The AlamarBlue assay was used to analyze cell viability on each biomaterial and on the control. Briefly, the culture medium was removed, each sample washed with PBS, then fresh medium with 5 % AlamarBlue was added to each well. The sample was incubated for 2.5 h at 37 °C, 5 % CO₂. Cells metabolic activity was detected by measuring the fluorescence at $\lambda = 590$ nm (excitation $\lambda = 540$ nm). Then, cells were re-fed with fresh culture medium and cultured at 37 °C in a humidified atmosphere with 5 % CO₂. The assay was carried out to evaluate the viability of cells attached and grown on the biomaterials, and control, at 24, 72, 144 and 216 h.

Assessment of cellular attachment

Human mesenchymal stem cells were loaded onto HA scaffolds, and onto TCPS as control, in 24-well culture plates ($\varnothing = 10$ mm) and cultured as described above. After 36 h of incubation, cell lysates were assayed using the Calbiochem PhosphoDetect FAK (pTyr397) ELISA kit (Merck KGaA, Darmstadt, Germany) to detect and

quantify the amount of focal adhesion kinase (FAK) protein phosphorylated at the tyrosin position 397 (Tyr397) following the manufacturer's instructions. Values were corrected for the protein concentration as determined by a colorimetric assay (BCA assay; Pierce, Rockford, IL).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation. Statistical analysis of cell-biology experiments, which were performed in triplicate, was carried out using SPSS (SPSS Inc., Chicago, IL) one-way analysis of variance (ANOVA) with Dunnet post-test analysis (* $p < 0.05$ and ** $p < 0.01$).

Results

Human mesenchymal stem cells were evaluated for the presence of specific surface antigens. The surface antigen profile matched with expected markers, according to the International Society for Cell Therapy guidelines. Indeed, cells expressed, as expected, CD29, CD44, CD71 and CD105 biomarkers (Fig. 1). The bone marrow aspirates gave fibroblast-like adherent cells, capable of forming colonies when seeded at low density (1,000 cells/cm²). Stem cells produced transient amplifying progeny and maintain a stem precursor cell during their asymmetric division, as described before [17]. Cell growth potential was maintained during 40 days of cultivation. The ability to produce cell generations correlates with self-renewal capacity, which is a specific feature of stem cells. The average mean doubling time, obtained from the same samples, was 3.2 days with a standard deviation of 0.5 days.

Purified hMSCs were loaded onto scaffolds as described in the MM section. High density cells suspension was placed in different wells containing a single scaffold. The number of cells seeded was determined by normalizing to the surface available for colonization per scaffold, with a density of 1,000 cells per cm². Incubation of 2 h was needed to allow the interaction between matrix and cellular adhesion proteins and for cell attachment to the substrate. Control (TCPS), which has a lower surface area, increased its metabolic activity during the assay period, showing a statistically significant difference ($p < 0.05$) between the metabolic activity detected at 36 and 216 h of incubation. Furthermore, Engipore scaffolds, characterized by inter- and intra-porosity, increased their metabolic activity during the assay with statistical differences ($p < 0.05$). The different behavior was toward Sintlife biomaterials; indeed the granular form increased metabolic activity to a statistically significant level ($p < 0.05$), while paste form maintained the same metabolic activity during the 9 days

Fig. 1 Surface antigenic profile of hMSCs obtained from bone marrow aspirates at the first culture passage. **a** CD235 and CD45 are considered as negative markers while **b** CD29, CD44, CD71 and CD105 are the positive markers

of the assay. A statistically significant difference in metabolic activity was found between TCP and scaffolds and between Engipore in the form of chips and Sintlife in paste form.

From viability data, we may postulate that Engipore chip scaffold better resembles the extracellular matrix features than Sintlife powder, offering an adequate support for cell adhesion and stimuli for proliferation. The quantification of pFAK relative amounts per scaffold may represent an indirect method for evaluating the strength of cell adhesion to biomaterials. The immuno-localization of pFAK-Tyr³⁹⁷ by immuno-staining with specific antibodies highlights its cytosolic distribution in the control samples. To quantify the relative amount of pFAK-Tyr³⁹⁷ per scaffold, we performed a sandwich ELISA test ($n = 3$). Results show that TCPS had the highest relative amount of pFAK-Tyr³⁹⁷ normalized to the total protein amount per sample, with a statistically significant difference ($p < 0.01$) toward each biomaterial. An evident difference was obtained between Engipore in the form of chips and Sintlife in paste form (Fig. 2) with a higher cytosolic level of phosphorylated FAK in hMSCs grown on Engipore biomaterial in the form of chips. Our data indicated that the two types of HA-derived materials induced different cellular responses, influencing focal adhesion assembling and downstream signaling pathways activation.

In the morphological analysis of hMSCs seeded on the biomaterials, the cells appeared well attached to the scaffold with several pseudopodia or elongations. Cells grown

Fig. 2 Relative amount per sample of pFAK (Tyr³⁹⁷) quantified by ELISA testing ($n = 3$). A difference between Engipore in the form of chips and Sintlife in paste form occurred. **TCPS exhibited the highest relative amount of pFAK (Tyr³⁹⁷) ($p < 0.01$)

on Engipore porous scaffolds showed a non-altered morphology and appeared well attached to the substrate with a high number of long pseudopodia in contact with the extracellular matrix. The amount of debris on the cell surface was low, according to its low release by these types of biomaterials. The same result was obtained with the Sintlife in the form of granules. Different behavior was registered toward Sintlife in paste form: cells attached to it showed a low number of shorter pseudopodia and a greater amount of debris on the cell surface due to the high release

Fig. 3 Cytoskeleton analysis by Phalloidin TRITC staining of hMSCs cultured on biomaterials. Actin filaments do not show alteration in the structural organization confirming the compatibility of assayed biomaterials, **a** Sintlife **b** control (TCPS). $\times 400$ magnification

of debris by this type of scaffolding when placed in culture medium (data not shown).

The cytoskeleton architecture is well organized and its integrity is not influenced by the cultivation of cells on the different scaffolds. Indeed, the cytoskeleton organization is similar to that of the cells grown on control. Actin fibers connect cell membranes and the cytoskeleton to the scaffold surface without any visible loss of structural displacement (Fig. 3).

Discussion

Spinal fusion is an established treatment for degenerative and traumatic lumbar spine disorders [2]. Recently, patients treated with surgical stabilization for degenerative lumbar spondylolisthesis had better clinical outcomes than patients treated nonsurgically [18]. Mechanisms to accelerate the fusion process and obviate the need for bone grafting will decrease patient morbidity and contribute to improved outcomes. The recent improvement in the knowledge of stem cell biology and biomaterials has allowed the setting up of new cellular models based on the use of precursor cells.

These cellular models can be employed to characterize *in vitro* the behavior of different biomaterials, which are used as bone void fillers or three-dimensional scaffolds. In our study, bone precursor cells from bone marrow aspirates of orthopedic patients were used to analyze bone stem cell behavior when cultured in close contact with selected scaffolds. The cell population was previously analyzed for the presence of specific surface antigens/biomarkers, as reported in earlier studies [16]. In our investigation, hMSCs maintained their self-renewal capacity during 5 culture

passages by increasing the cumulative number of cell doublings. This capacity was assayed in three samples from different donors. The results confirmed that the cell population isolated from bone marrow aspirates showed characteristic stem cell features. The mean doubling time of hMSCs, isolated from three samples and grown for 40 days, is in agreement with an earlier report [19]. Furthermore, our data on cell viability are in agreement with the results of another study, which was carried out with MC3T3-E1 preosteoblasts grown on different HA-derived scaffolds [20].

Phosphorylation of focal adhesion kinase, involved in the assembling of focal adhesion, induces a cellular response that leads to cell survival, proliferation and differentiation via several cellular signal pathways and is also responsible for the maintenance of cell adhesion to the substrate. Our data obtained by ELISA quantification add molecular evidence to the differences observed in the metabolic assay. Indeed, Engipore in the form of chips shows a pFAK-Tyr³⁹⁷ relative amount higher than Sintlife biomaterial in paste form as observed in the metabolic activity assay. As reported previously with an osteoblastoid cellular model [21] a rough surface has more ability to induce the activation of FAK in response to integrin binding to the substrate. Despite its rough surface, Sintlife in paste form is characterized by a compact microstructure and the absence of porosity suitable for cell hosting and fluid flow, while the same type of biomaterial in granular form exhibits an inter-granular porosity. The different physical features of the two forms of the Sintlife material, in terms of the presence or absence of colonizable niche and fluid flow channels, may explain the lower relative amount of pFAK-Tyr³⁹⁷ detected in the cells grown on Sintlife in form of paste and thus their constant metabolic

activity. Biomaterials assayed herein show a good level of cytocompatibility as suggested by the viability test. Besides this, hMSCs cytoskeleton organization is not affected by biomaterial chemical and physical features. Although hMSCs morphology is influenced by the scaffold physical structure, HA materials did not induce cytotoxic effects. However, the comparative analyses of biomaterials showed several differences in their performances. Indeed, the viability test and FAK ELISA assay indicate that high porous scaffolds represent the best microenvironment. In addition, these data suggest that an important role is played by intra- and inter-porosity, with the best performance achieved when these parameters are combined together in the same biomaterial. Cell adhesion appears positively influenced by physical structure with low solubility as determined by the pFAK-Tyr³⁹⁷ relative amounts per scaffolds.

Data collected from the analysis described showed that both biomaterials could be good candidate for substitute bone grafts in spine fusion. Engipore chips have a greater “homing” potential than Sintlife nanopowder, although both biomaterials do not show any cytotoxic effect when employed as substrates in cell culture. Further in vivo investigations are needed to clarify the influence of magnesium ion when included in the chemical structure of HA scaffolds.

Conclusions

In conclusion, the results of this study support the benefits of the hMSCs and biomaterials synergy to facilitate spinal fusion. This approach exhibits the benefits of bone marrow grafts without the associated morbidity or recognized potential for disease transmission. Thus, the use of hMSCs in combination with biomaterials for spinal fusion as well as general bone regeneration merits further investigation as a therapeutic modality.

The cellular model used in our study, based on hMSCs obtained from the bone marrow aspirate of orthopedic patients/donors, suggests that this in vitro model is an useful tool for investigating cytocompatibility and biological features of HA-derived scaffolds.

In our study, we found that the biomaterials assayed differently influence cell behavior. Indeed, we observed higher proliferation and focal adhesion kinase activation toward Engipore chip substrate, suggesting its better osteoconductive potential than Sintlife.

Investigations with hMSCs could represent an accurate and personalized way to evaluate the suitability of each type of material toward patients and allowing clinical implantation of the scaffolds which show in vitro the required properties for successful bone tissue regeneration,

considering the importance of finding suitable substitutes for spine fusion.

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Conflict of interest None.

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