Titanium and tantalum as mesenchymal stem cell scaffolds for spinal fusion: an in vitro comparative study

Juan F. Blanco · Fermin M. Sánchez-Guijo ·
Soraya Carrancio · Sandra Muntion ·
Jesus García-Briñon · Maria-Consuelo del Cañizo

Received: 15 February 2011 / Revised: 24 March 2011 / Accepted: 29 June 2011 / Published online: 16 July 2011
© Springer-Verlag 2011

Abstract

Introduction In the last few years, great interest has been focused on tissue engineering as a potential therapeutic approach for musculoskeletal diseases. The role of metallic implants for spinal fusion has been tested in preclinical and clinical settings. Titanium and tantalum have excellent biocompatibility and mechanical properties and are being used in this situation. On the other hand, the therapeutic role of mesenchymal stem cells (MSC) is extensively explored for their multilineage differentiation into osteoblasts.

Objectives In vitro comparison of titanium and tantalum as MSCs scaffolds.

Material and methods In the present study, we have compared the in vitro expansion capacity, viability, immunophenotype (both explored by flow cytometry) and multi-differentiation ability of MSC cultured in the presence of either titanium or tantalum fragments. The adherence of MSC to either metal was demonstrated by electron microscopy.

Results Both metals were able to carry MSC when transferred to new culture flasks. In addition, our study shows that

culture of MSC with titanium or tantalum improves cell viability and maintains all their biological properties, with no significant differences regarding the metal employed.

Conclusion This would support the use of these combinations for clinical purposes, especially in the spinal fusion and reconstruction setting.

Keywords Titanium scaffold · Tantalum scaffold · Mesenchymal stem cells · Tissue engineering

Introduction

Tissue engineering includes all the procedures that use cells together with a biocompatible scaffold to repair or substitute a defined damaged space within any organ or tissue. The latter not only plays a supportive role but also may directly contribute to the biological action. In addition, several cytokines or growth factors can be added to both cells and scaffolds in order to enhance the final effects [1].

J. F. Blanco (✉)
Department of Orthopedics, Servicio de Traumatología y CO,
Hospital Universitario de Salamanca, Paseo de San Vicente 58,
37007 Salamanca, Spain
e-mail: jfblanco@usal.es

J. F. Blanco · F. M. Sánchez-Guijo · S. Carrancio ·
S. Muntion · J. García-Briñon · M.-C. del Cañizo
Centro en Red de Terapia Celular y Medicina Regenerativa de
Castilla y León, Red Nacional de Terapia Celular (TerCel),
Salamanca, Spain
e-mail: ferminsg@usal.es

S. Carrancio
e-mail: intersori@hotmail.com

S. Muntion
e-mail: smuntion@yahoo.es

J. García-Briñon
e-mail: jgb@usal.es

M.-C. del Cañizo
e-mail: concarol@usal.es

F. M. Sánchez-Guijo · M.-C. del Cañizo
Department of Hematology, Hospital Universitario de
Salamanca, Paseo de San Vicente 58, 37007 Salamanca, Spain

S. Carrancio · S. Muntion
Cell Therapy Lab, Hospital Universitario de Salamanca,
Paseo de San Vicente 58, 37007 Salamanca, Spain

J. García-Briñon
Department of Cell Biology, Facultad de Medicina,
Universidad de Salamanca, Salamanca, Spain

Mesenchymal stem cell (MSC) research is currently an exciting area of interest, since these cells have the ability to differentiate into several cell types, including osteoblasts, chondrocytes and adipocytes. Another advantage of MSC is the fact that they can be isolated and expanded from several sources, especially from bone marrow (BM) and fat. For all those reasons, they are extensively assessed in musculoskeletal research [14, 16, 21]. In this regard, they have been tested for spinal fusion [10, 11, 13, 15, 18] or for bone defects treatment [17]. In the latter manuscript, a calcium phosphate scaffold was used. This approach is controversial, since this type of ceramic support may be quite fragile for being used in a high load bone segment [14].

The use of metallic implants has been increasing in the last few decades either for osteo-synthesis or for arthroplasty, as for spinal fusion procedures. This biocompatible metallic materials, although give instant support to bone segment, cannot be reabsorbed and substituted by new bone formation. Nevertheless, they have a tridimensional structure and porosity that favors bone anchorage [4, 23].

Titanium and tantalum are two metals highly used in the clinical setting because their excellent biocompatibility and their mechanical properties. There are currently several commercial devices for intersomatic spinal fusion, either for cervical and lumbar segments, based on both of them. Although results of spinal fusion techniques using this technology show better fusion rates than the classical procedures, like posterolateral arthrodesis [25], and even though anterior arthrodesis is still the gold-standard for cervical fusion, there is still a chance of failure [7]. In other cases, where a more complex approach (e.g., corpectomy) has to be performed, a strong fixation of metallic implants is needed to obtain an optimal reconstruction and also to ensure safety.

As it was initially reported by Bruder et al. [5], loading metallic devices with MSC enhances bone reconstruction when compared to non-cellular devices. Thus, Gastaldi et al. [12] support the use of titanium devices with progenitor cells in order to regenerate damaged bone tissue.

In the present work, we have comparatively analyzed the multiparametric “in vitro” characteristics of MSC co-cultured with both commercially available titanium and tantalum fragments that are highly employed in spinal fusion procedures. The working hypothesis was that MSC would adhere to either titanium or tantalum and this construct would not hold back any of their in vitro properties. This would be of great impact to support their rationale for the clinical studies assessing the use of this tissue-engineering approach.

Materials and methods

An outline of all the experiments performed in this study is depicted in Fig. 1. All the procedures specified below were

approved by the Ethical Committee of the Hospital Universitario de Salamanca and were also in accordance with the Helsinki Declaration of 1975, as revised in 2000, and informed consent was obtained from all subjects whose samples were included in the current study.

Scaffolds

Both commercially available titanium and tantalum sources for intersomatic spinal fusion were used in the present study. Titanium was acquired from Synthes Inc. and the trade name is Plivipore®. Tantalum trade name was Trabecular metal-TM® from Zimmer Inc. (Fig. 2). In both cases, fragments from the original piece were obtained by cutting and sterilized using an autoclave.

Samples, MSC isolation and expansion

For the present study, 10 mL of BM were obtained by iliac crest aspiration from five voluntary donors, after specific informed consent was obtained. Median age was 48 years (range 34–59 years) and male/female ratio was 4/1. MSC from BM were isolated as previously described [6, 19]. Briefly, low-density mononuclear cells were isolated by Ficoll-Paque (Biochrom KG, Berlin, Germany) and plated at a cell density of 10^6 MNC/cm² on a plastic surface in DMEM (Gibco BRL, Paisley, UK) culture medium containing 10% of pre-selected fetal calf serum (FCS; BioWhittaker, Verviers, Belgium). Culture flasks were maintained in a humidified incubator at 37°C with 5% CO₂. Twice a week, adherent cells were fed by complete replacement of the medium (and non-adherent cells subsequently washed out). When the layer was 80% confluent, the culture was trypsinized and cells were subcultured onto two or three culture flasks (this is termed as a “cell passage”) at a cell density of 2.5×10^3 cells/cm². Cells underwent three passages in culture, to ensure that all contaminating hematopoietic cells had been totally washed out of the flask. Cell numbers obtained after each passage and the time to reach an 80% confluent layer was recorded in each comparative expansion.

Flow cytometric immunophenotyping

For the immunophenotypic characterization of MSC by flow cytometry, detached cells were washed and resuspended in phosphate buffer solution (PBS), and incubated with the following monoclonal antibodies directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Cyanin-5 (PCy5), peridinin-chlorophyll-protein (PerCP) or allophycocyanin (APC): CD90-FITC, CD14-FITC, CD73-PE, CD106-PE, CD166-PE, HLA-DR-PerCP, CD45-PCy5, CD34-APC and CD19-APC all from

Fig. 1 Outline of experimental design

Fig. 2 Commercially available titanium and tantalum sources were used in the present study. Titanium (**a**) was acquired from Synthes Inc., and the trade name is Plivipore®. Tantalum (**b**) trade name was Trabecular metal-TM® from Zimmer Inc. Both were fragmented by cutting

BDB (San Jose, CA, USA) and CD105-FITC from ImmunoStep (Salamanca, Spain). Isotype IgG control antibodies (BDB) were used in each case. Cells were then washed and acquired in a FACSCalibur flow cytometer (BDB). At least

10^5 events were collected per sample. Immunophenotypic analysis was performed with the CellQuest and Paint-A-Gate Pro software (BDB).

Multilineage differentiation of MSC

In order to demonstrate the multilineage differentiation potential of MSC in each experimental group, adipogenic, osteogenic and chondrogenic differentiation was induced as previously described [19].

For osteogenic differentiation, fourth passage MSC were plated at 5×10^3 cells/cm² in a 9.6 cm² slideflask (Nunc, Denmark), grown up to 80% confluence and incubated in osteogenic medium (NH Osteodiff Medium, Miltenyi Biotec, Germany). The medium was replaced every 3–4 days. After 10 days, cultures were washed with PBS, fixed in a solution of ice-cold 70% ethanol for 10 min. For PA staining, slides were fixed in 100% methanol for 5 min, and then washed with PBS and then incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT, Sigma-Aldrich) during 15–30 min, strictly following manufacturer's instructions.

For adipogenic induction, MSC from both sources were cultured in NH Adipodiff Medium (Miltenyi Biotec,

Germany) under the same conditions (37°C with 5% CO₂) for 21 days. For oil-red-O staining, slides were fixed 3.7% formaldehyde during 2 min, and then washed. Afterward, slides were incubated with oil-red-O (Merck, Darmstadt, Germany) during 1 h at room temperature.

A chamber slide that contained MSC growing in expansion medium (DMEM with 10% FCS, as described before) without any differentiation cocktail was used as control for osteogenic and adipogenic differentiation.

All sets of slides were washed with distilled water and mounted for optical microscopic analysis.

For chondrogenic differentiation, 5×10^5 cells were placed in a 15 mL polypropylene tube and centrifuged. The pellet was cultured at 37°C and 5% CO₂ in 500 µL chondrogenic medium (NH Chondrodiff Medium, Miltenyi Biotec, Germany), following manufacturer's protocol. The medium was changed every 3–4 days. After 21 days of culture, pellets were fixed by immersion in cold (4°C) 4% paraformaldehyde and cryoprotected by immersion overnight in 30% sucrose in phosphate buffer 0.1 M pH 7.4. Then, 5 µm thick sections were cut in a cryostat (Leica CM1850) and serially collected on gelatin-coated slides. After several washes in phosphate buffer sections were processed for toluidine blue staining. Slides were placed on a hot plate and sections were covered with drops of staining solution (1% aqueous toluidine blue and 1% aqueous sodium tetraborate) for 5 min. After that, sections were rinsed several times in distilled water, quickly dehydrated through 95% and two changes of 100% ethanol (several dips each since stain fades quickly in alcohol), cleared in xylene and coverslipped with a resinous mounting medium (Entellan, Merck).

Analysis was performed in an Olympus Provis photomicroscope (Olympus Europe GmbH, Hamburg, Germany), and images were taken and digitalized with a DP-70 digital camera (Olympus Optical CO, GmbH, Hamburg, Germany) adapted to a computer, using the DP Controller v. 1.2.1.108 and the DP Manager v. 1.2.1.107 software (Olympus Optical CO., GmbH, Hamburg, Germany).

Cell adhesion study and expansion of MSC with metal fragments

All the metal fragments were placed in the standard MSC expansion culture medium described above, and human MSC were added. Cell adhesion to either titanium or tantalum fragments were assessed by optical microscopy and electron microscopy after 3 and 14 days of contact, using a Zeiss DSM 940 scanning electron microscope.

As it has been mentioned, metallic fragments of either titanium or tantalum were incubated with 5×10^5 MSC in 100–200 µL total volume of expansion medium during

3 days. Afterward, metallic fragments “carrying” adhered MSC were placed in an acellular culture flask with MSC expansion media that was replaced twice weekly, until 80% confluence was obtained. As it has been depicted in Fig. 1, cells were then evaluated for expansion capacity, viability, immunophenotyping and multilineage differentiation. Since most of the techniques have been already described, we will describe next only the viability studies.

Cell viability and apoptosis

To assess viability and/or apoptosis, MSCs were marked with a FITC-conjugated anti-CD90 monoclonal antibody (from BD), as described earlier in this manuscript, to further identify them during the analysis. Cells were then incubated with Annexin-V and 7-actinomycin (7-AAD) that will recognize cells in apoptosis or dead cells, respectively. For this purpose, the commercially available BD apoptosis kit was employed, strictly following manufacturer's instructions. Sample acquisition was performed in a FACSCalibur flow cytometer using the CellQuest software, all from BD. Samples were analyzed using the Infinicyt software (Cytognos, Salamanca, Spain), for the percentage of viable cells (double annexin-V and 7-AAD negative cells).

Results

Expansion of MSC with scaffolds

In all cases, MSCs were “in vitro” expanded up to third passage in culture when cultured in the presence of titanium, tantalum or with no metal. The expansion rate was slightly faster for cells expanded with titanium that formed an 80% confluent layer at a median time of 14 days, compared to those expanded with tantalum, that needed a median of 17 days to reach that confluence. Cells with no metal reached the confluence also after a median time of 2 weeks. In all cases, MSCs exhibited their typical spindle shape morphology (Fig. 3).

Adhesion imaging

MSC adhered to either titanium or tantalum occupying the porous structures, as it can be observed with the inverted microscope (Fig. 4). Regarding scanning electron microscope, as it is depicted in Figs. 5 and 6, MSC did adhere to either metallic surface or displayed cytoplasmic prolongations for anchorage to the porous structure. Although the numbers of adhered MSC was not quantified, they increased with time.

Fig. 3 Cell expansion and cell morphology in both media. (**a** MSC-titanium. **b** MSC-tantalum). The MSC (mesenchymal stem cells) exhibited their typical spindle shape morphology in both media

Fig. 4 Inverted optical microscope. Cell adhesion in both scaffolds. Cells are attached in the porous structure. (**a** MSC-titanium, **b** MSC-tantalum)

Fig. 5 Scanning electron microscopy. MSCs attached to the Tantalum. The cells exhibit cytoplasmic extensions to attach to porous structure

Viability analysis

Interestingly, the median percentage of viable MSC assessed by annexin-V and 7-AAD staining by flow cytometry was significantly higher in the groups cultured in the presence of either titanium or tantalum fragments compared to MSC expanded in the standard conditions without metals, as it is shown in Fig. 7.

Immunophenotypic analysis

Flow cytometric analysis of MSC expanded after being adhered to either titanium or tantalum showed that these cells maintain the standard immunophenotypic profile in accordance to the International Society for Cellular

Therapy definition criteria, without differences regarding any of the groups analyzed, as it is depicted in Fig. 8.

Multilineage differentiation analysis

In all cases, MSCs adhered to either titanium or to tantalum and further expanded in vitro were able to differentiate into osteoblasts, adipocytes and chondrocytes under specific culture conditions, without differences between groups.

Discussion

Bone repair by tissue-engineering technology requires a cellular source with the ability of differentiation into bone

Fig. 6 Scanning electron microscopy. MSCs attached to the titanium. The cells exhibit cytoplasmic extensions to attach to porous structure

Fig. 7 Viability analysis by flow cytometry. The viability was significantly higher in the groups cultured in the presence of either titanium or tantalum fragments compared to MSC expanded in the standard conditions without metals

together with a scaffold that allows the adhesion and transport of these cells to the injured tissue. In addition, the scaffold should also have several other characteristics that may enhance the reparative process. Several biomaterials have been tested for this purpose, most of them are resorbable after the repairing process has concluded. In addition, they often have a porous three-dimensional structure that favors cell adhesion and nutrition [2, 11, 17, 24]. Materials tested in the current work have demonstrated an excellent biocompatibility, and provided structural resistance [4, 8, 26]. MSC can be isolated from several sources and display multilineage differentiation ability that includes osteoblastic differentiation. Apart from this

property, they display an established immunophenotype and are in vitro expanded based on their property of adherence to plastic surfaces pattern [3, 9].

Most studies published to date in this setting are focussed on bone repair by combination of progenitor cells with several biomaterials, especially calcium phosphate compounds based on their similarities with the mineral bone phase and their resorbability. The few works that explore interactions of titanium and tantalum with MSC are mainly centred on assessing the effects on the structural architecture, especially the surface [20, 22, 26].

To the best of our knowledge, our study is the first that specifically and comparatively explores the viability and

Fig. 8 Immunophenotypic analysis by flow cytometry. MSC expanded after being adhered to either titanium or tantalum showed that these cells maintain the standard immunophenotypic profile in

accordance to the International Society for Cellular Therapy definition criteria, without differences regarding any of the groups analyzed

the multiparametric effects of both metals on MSC obtained from bone marrow. Recently, Gastaldi et al. [12] have evaluated the osteoblastic differentiation capacity of adipose-tissue-derived MSC cultured with porous titanium. They show that cells adhere to the metallic surface and are able to differentiate into osteoblasts. Our work demonstrated not only osteoblastic but also multilineal differentiation capacity. We have compared the titanium effects to those of tantalum showing that co-culturing MSC with both metals increases their viability measured by flow cytometry compared to cells that are cultured without metals as well as preserves their immunophenotypic and differentiation patterns. We have not found any significant difference between both metals, although the expansion rate of MSC cultured with titanium was faster. An interesting finding is the fact that both titanium and tantalum have the ability of tightly carrying MSC on their surfaces and adhered cells can repopulate “in vitro” the culture flask when the appropriate expansion medium is present. This would support the idea that MSCs can be safely embedded in either metal before implantation for spinal fusion and reconstruction purposes.

This study has some limitations that mainly include quantification of the number of cells directly attached to each metallic fragment, although an indirect estimation of this parameter has been the number of expanded cells “carried” by titanium or tantalum fragments. This, again, is mostly an estimation of the number of cells anchored to the surface of the fragments, since it is difficult to assess the proportion of the cells carried in the inner part of each fragment.

Conclusions

Our study shows that culture of MSC with titanium or tantalum improves cell viability and maintains all their biological properties. Both scaffolds are capable of delivering human mesenchymal stem cells without causing a loss of differentiation capabilities. This would support the use of these combinations for clinical purposes, especially in the spinal fusion procedures and reconstructive techniques.

Conflict of interest None.

References

- Atala A (2004) Tissue engineering and regenerative medicine: concepts for clinical application. *Rejuvenation Res* 7:15–31
- Anselme K (2000) Osteoblast adhesion on biomaterial. *Biomaterials* 21:667–681
- Barry FP, Murphy JM (2004) Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 36:568–584
- Bobyn JD, Steckpool G, Hacking SA, Tanzer M, Krygier JJ (1999) Characteristics of bone ingrowth and interface mechanics of a new porous tantalum biomaterial. *J Bone Joint Surg Br* 81:907–914
- Bruder SP, Kraus KH, Goldberg VM, Kadiyala S (1998) The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone J Surg Am* 80:985–996
- Carrancio S, López-Holgado N, Sanchez-Guijo MF et al (2008) Optimization of mesenchymal stem cell expansion procedures by cell separation and culture conditions modification. *Exp Hematol* 36:1014–1021

7. Cassinelli EH, Wallach C, Hanscom B, Vogt M, Kang JD (2006) Prospective clinical outcomes of revision fusion surgery in patients with pseudoarthrosis after posterior lumbar interbody fusions using stand-alone metallic cages. *Spine J* 6:428–434
8. D'Angelo F, Murena L, Campagnolo M, Zatti G, Cherubino P (2008) Analysis of bone ingrowth on a tantalum cup. *Indian J Orthop* 42:275–278
9. Dominici M, Le BK, Mueller I et al (2006) Minimal criteria for defining multipotent mesenchymal stem cells. The international society for cellular therapy position statement. *Cytotherapy* 8:315–317
10. Fu TS, Chen WJ, Chen LH, Lin SS, Liu SJ, Ueng SW (2009) Enhancement of posterolateral lumbar spine fusion using low-dose rhBMP-2 and cultured marrow stromal cells. *J Orthop Res* 27:380–384
11. Gan Y, Dai K, Zhang P, Tang T, Zhu Z, Lu J (2008) The clinical use of enriched bone marrow stem cells combined with porous beta-tricalcium phosphate in posterior spinal fusion. *Biomaterials* 29:3973–3982
12. Gastaldi G, Asti A, Scaffino MF, Visai L, Saino E, Cometa AM, Benazzo F (2010) Human adipose-derived stem cells (hASCs) proliferate and differentiate in osteoblast-like cells on trabecular titanium scaffolds. *J Biomed Mater Res A* 94:790–799
13. Gottfried ON, Dailey AT (2008) Mesenchymal stem cell and gene therapies for spinal fusion. *Neurosurgery* 63(3):380–391
14. Kraus KH, Kirker-Head C (2006) Mesenchymal stem cells and bone regeneration. *Vet Surg* 35(3):232–242
15. Koga A, Tokuhashi Y, Ohkawa A, Nishimura T, Takayama K, Ryu J (2008) Effects of fibronectin on osteoinductive capability of fresh iliac bone marrow aspirate in posterolateral spinal fusion in rabbits. *Spine* 33:1318–1323
16. Le Blanc K, Pittenger M (2005) Mesenchymal stem cells: progress toward promise. *Cytotherapy* 7:36–45
17. Marcacci M, Kon E, Moukhachev V, Laurokov A, Kutepof S, Quarto R, Mastrogiacomo M, Cancedda R (2007) Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. *Tissue Eng* 13:947–955
18. Nakajima T, Iizuka H, Tsutsumi S, Kayakabe M, Takagishi K (2007) Evaluation of posterolateral spinal fusion using mesenchymal stem cells: differences with or without osteogenic differentiation. *Spine* 32:2432–2436
19. Sanchez-Guijo F, Blanco JF, Cruz G et al (2009) Multiparametric comparison of mesenchymal stromal cells obtained from trabecular bone by using a novel isolation method with those obtained by iliac crest aspiration from the same subjects. *Cell Tissue Res* 336:501–507
20. Sjöström T, Dalby MJ, Hart A, Tare R, Oreffo ROC, Su B (2009) Fabrication of pillar-like titania nanostructures on titanium and their interaction with human skeletal stem cells. *Acta Biomater* 5:1433–1441
21. Stiehler M, Seib FP, Rauh J, Goedecke A, Werner C, Bornhäuser M, Günther KP, Bernstein P (2010) Cancellous bone allograft seeded with human mesenchymal stromal cells: a potential good manufacturing practice-grade tool for the regeneration of bone defects. *Cytotherapy* 12:658–668
22. Stiehler M, Lind M, Mygind T et al (2008) Morphology, proliferation, and osteogenic differentiation of mesenchymal stem cells cultured on titanium, tantalum, and chromium surfaces. *J Biomed Mater Res A* 86:448–458
23. Wazen RM, Lefebvre LP, Baril E, Nanci A (2010) Initial evaluation of bone ingrowth into a novel porous titanium coating. *J Mater Res B Appl Biomater* 94:64–71
24. Weinad C, Pomerantseva I, Neville CM, Gupta R et al (2006) Hydrogel-beta-TCP scaffolds and stem cells for tissue engineering bone. *Bone* 38:555–563
25. Xiuxin H, Yue Z, Cui C, Yajun W (2009) A meta-Analysis of circumferential fusion versus instrumented posterolateral fusion in the lumbar spine. *Spine* 34:E618–E625
26. Zhou C, Zhan E, Lin M, Zeng S (2008) Preparation, microstructure and mechanical properties of porous titanium sintered by Ti fibres. *J Mater Sci Mater Med* 19:401–405