Porous poly (L-lactic acid) scaffolds are optimal substrates for internal colonization by A6 mesoangioblasts and immunocytochemical analyses

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In this study, mouse mesoangioblasts were seeded onto bidimensional matrices within three-dimensional porous scaffolds of poly (L-lactic acid) (PLLA), in the presence or absence of a type I collagen coating. The cells were observed under a scanning electron microscope and tested for their adhesion, survival and proliferation. Immunolocalization of heat shock protein (Hsp) 70, an abundant and ubiquitous intracellular protein in these cells, was also performed in sectioned cell-containing scaffolds under a confocal fluorescence microscope to determine if *in situ* analysis of intracellular constituents was feasible. The data show that PLLA films allow direct cell adhesion and represent an optimal support for cell growth, and that the internal surfaces of PLLA polymeric sponges can be colonized by mesoangioblasts, which can be submitted for *in situ* confocal microscopic analyses for possible monitoring of time-dependent expression of differentiation markers.

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1. Introduction

Regenerative medicine is a multidisciplinary branch of medicine aimed at the replacement and/or repair of diseased tissues via cell therapy and tissue engineering. The latter has arisen from the rapid advancement in the fields of both cellular and molecular biology and chemical/mechanical engineering, leading to the design of novel biomaterials for scaffold production. The major function of scaffolds is to provide a temporary support for tissue structures. Scaffolds are largely biodegradable, synthetic, three-dimensional (3D) supports emulating the extracellular matrix and containing an interconnected pore network that guides cell adhesion, proliferation and differentiation. Thus, a regenerated tissue exhibiting a well-integrated structure (e.g. Seunarine *et al.* 2006) is reproduced.

Scaffolds used in tissue engineering applications should demonstrate compatible biological and physical properties that match physiological conditions *in vitro* and *in vivo*. When compared to other materials, biodegradable synthetic polymers offer several key advantages for developing scaffolds capable of inducing tissue growth, such as the ability to tailor their mechanical properties and degradation kinetics to suit various applications, the versatility of fabrication of scaffolds of varying shape with desired pore morphological features, and the option of exposing specific and selected chemical functional groups that can promote tissue reconstitution (Gunatillake *et al.* 2006). For these reasons, a number of studies have focused on the standardization and production of biodegradable polymers for various biomedical applications, such as bone repair (Eglin and Alini 2008; Young and Ho 2008), peripheral nerve or vascular growth, and bio-artificial devices for drug delivery (Wang *et al.* 2005).

In recent years, a large number of tissue engineering experiments have been carried out using stem cells for regeneration applications. In particular, embryonic stem cells represent a promising source due to their ability

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Abbreviations used: HMGB1, high-mobility group box 1; Hsp, heat shock protein; PBS, phosphate-buffered saline; PLLA, poly (L-lactic acid); SDF1, stromal derived factor 1; SEM, scanning electron microscopy; TNF, tumour necrosis factor

to give rise to all somatic cell lineages (Gunatillake and Adhikari 2003). Moreover, a number of reports have shown that when mouse embryonic stem cells are cultured in 3D scaffolds, the expression of differentiation marker genes is increased (e.g. Carpizo *et al.* 2008; Lin *et al.* 2007). Among the synthetic biomedical materials used for tissue engineering, poly (L-lactic acid) (PLLA) polymers represent a resorbable, biocompatible, non-toxic support with consolidated applications in dermatological practice and in the management of soft tissue augmentation (Perry 2004; Vochelle 2004; Tsuji 2005).

Mesoangioblasts are stem cells recently isolated from the aorta of mammalian embryos (Minasi *et al.* 2002). These cells have been characterized as a population of vessel-associated multipotent cells co-expressing both endothelial and myogenic markers. *In vitro*, they are capable of self-renewal, retaining multipotency and differentiating into various mesodermal cell types and tissues. *In vivo*, they show the ability to migrate towards sources of released high-mobility group box 1 (HMGB1) protein, to proliferate (Palumbo *et al.* 2004; Palumbo *et al.* 2007) and colonize damaged tissues when previously exposed to stromal derived factor 1 (SDF1) or tumour necrosis factor (TNF) (Galvez *et al.* 2006). Thus, they represent a promising cell system for the study of the cellular and molecular mechanisms of tissue repair. Interestingly, mesoangioblasts have been found capable of reducing post-infarction left ventricular dysfunction when injected into the ventricular chambers of experimental models of myocardial infarction (Galli *et al.* 2005) and ameliorating dystrophic skeletal muscle through intra-arterial delivery in dystrophic mouse and dog models (Sampaolesi *et al.* 2006). This highly ductile population of stem cells capable of self-renewal, differentiation, colonization and/or proliferation appears to be a suitable model system for the study of experimental tissue engineering within 3D scaffolds.

The goal of this study was to determine the capacity of mouse A6 mesoangioblast stem cells to adhere, survive and proliferate onto bidimensional (2D)-matrices and within 3D-porous scaffolds of PLLA. The results obtained show that PLLA films allow cell adhesion and represent an optimal support for cell growth, and that the internal surfaces of PLLA polymeric sponges can be colonized by mesoangioblasts for observation by *in situ* confocal microscopic analyses for possible monitoring of time-dependent expression of differentiation markers.

2. Materials and methods

2.1 *Cells*

The A6 cell line was grown in DMEM (Sigma, St Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS, Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/l amphotericin B, at 37°C in a 5% CO_2 atmosphere.

2.2 *PLLA film preparation and cell growth assay*

To obtain a 2D-PLLA film on glass slides, slides were covered with a solution of 0.1% w/w PLLA in dioxane and placed under vacuum at room temperature overnight to evaporate the solvent. To completely remove the dioxane, slides were subsequently rinsed in 1X phosphate-buffered saline solution (PBS; 68 mM NaCl, 34 mM KCl, 1.4 mM Na_2HPO4, 0.44 mM $CaCl_2$, 0.24 mM $MgCl_2$ and 0.75 mM KH_2PO_4). A set of PLLA-coated slides was covered with an acidic type I collagen solution (50 μg/ml in 0.02 N CH_3COOH) for 48 h and then rinsed with fresh medium to neutralize the substrate. Finally, the slides were sterilized with 70% ethanol overnight. A batch of PLLA films was coated with type I collagen for 48 h. A suspension of approximately 2.5×10^4 cells in FBS-containing DMEM was seeded onto plain, PLLA-coated, or PLLA/collagen-coated slides. Cell number was evaluated after 24, 48 and 72 h from plating by counting in a Bürker chamber after cell trypsinization. Cell growth rate and doubling time in the various culture conditions were determined according to the methods of Luparello (1990).

2.3 *PLLA scaffold preparation*

PLLA scaffolds were prepared according to the method of Pavia (2008). A homogeneous ternary solution of PLLA, dioxane and water was prepared, with a constant dioxane-to-water weight ratio of 87:13. The concentration of PLLA chosen was 4% wt/wt. The solution, initially kept at 60°C, was hot-poured into an aluminium disc-shaped sample holder, with a diameter of 60 mm and a thickness of 2 mm. The temperature was then rapidly lowered to a value within the unstable region (30°C) for 30 min by pool immersion of the sample holder into a thermostatic water bath. A quench by pool immersion in an ethyl alcohol bath at a temperature of –20°C for 5 min was then performed to freeze the as-obtained 3D structure. The resulting foams were then subjected to washing in deionized water for 24 h and drying at 20°C under vacuum overnight to remove any remaining solvent.

2.4 *Cell culture into the scaffolds*

The scaffolds were cut into small squares (approximately 1 cm^2), individually placed in Petri dishes (Ø = 6 cm), sterilized with 70% ethanol overnight, and rinsed extensively with an excess of 1X PBS. A cell suspension of 5×10^5 cells/ml (cells in the exponential growth phase) in FBS-containing

DMEM was recovered in a 1 ml syringe and inoculated, applying low pressure, in five regions of the scaffolds to assure homogeneous cell distribution. Ten minutes after cell inoculation, so that the medium with the cells was distributed throughout the scaffold, the Petri dishes were filled with 10 ml of fresh medium. The cell-containing scaffolds were maintained at 37°C under a 5% CO_2 atmosphere for 30 min. Subsequently, the fresh culture medium was replaced to remove some cells that were not inside the scaffold. After 24 or 48 h of culture, the cells were prepared for microscopic observation.

2.5 *Optical microscopy*

Cell-containing scaffolds were treated with 5% v/v glutaraldehyde in DMEM to fix cells attached to the internal surfaces of the PLLA scaffolds. After fixing, the cells were washed exhaustively with 1X PBS and stained for 5 min. The cytological dyes used were toluidine blue, methylene blue, Nile blue and Janus green (1% in 1X PBS), and Giemsa stain. The scaffolds were then rinsed twice for 30 min in dye solvent to remove excess stain. The stained cells attached within the 3D supports were observed under a light stereomicroscope (Leica MS 5 with Leica DC 80 Digital Camera) in 10 scaffold cross-sections obtained with a surgical blade.

2.6 *Scanning electron microscopy (SEM)*

For SEM observations, scaffold cross-sections were fixed as described above, rinsed in 1X PBS, and dehydrated with increasing concentrations of ethanol. Dehydrated samples were gold sputter-coated and observed under a Philips 505 SEM.

2.7 *Immunofluorescence assays*

A6 cells were seeded into the scaffold as described above. After 24 or 48 h, the cell-seeded scaffolds were washed with 1X PBS and fixed in 3.7% formaldehyde for 15 min. The cells were then washed three times with 1X PBS and permeabilized with 0.1% Triton-X 100 for 5 min. Permeabilized cells were washed with 1X PBS and incubated overnight at 4°C with monoclonal mouse anti-Hsp70 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA/USA; working dilution 1:500), followed by the secondary fluorescein-conjugated anti-mouse antibody (Amersham, Little Chalfont, UK; working dilution 1:50). The scaffolds were mounted with 1,4-diazobicycloro[2-2-2]octane and observed under an Olympus FV300 laser-scanning confocal microscope equipped with Argon (488 nm) and Helium/Neon (543 nm) lasers with a PlanApo 60× 1.40 oil immersion lens, and scanned at 1024 × 1024 pixel resolution.

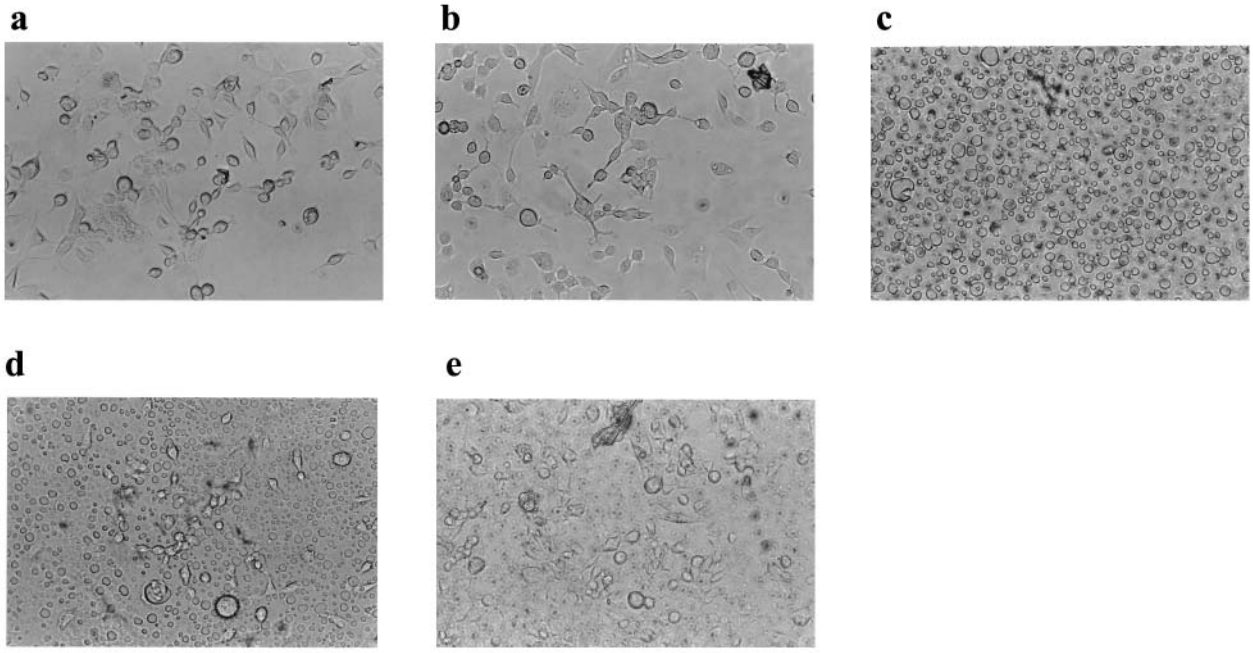

Figure 1. Morphology of mesoangioblast cells seeded on glass slides with or without 2D-PLLA and collagen coating, observed under the light microscope. **(a)** Cells on a glass slide. **(b)** Cells on a collagen-coated glass slide. **(c)** Aspect of glass slide coated with PLLA in the absence of cells. **(d)** Cells on a glass slide coated with a 2D-PLLA film. **(e)** Cells on a glass slide coated with both PLLA and collagen. (**a**–**e**) Magnification 10×.

2.8 *Statistical analyses*

Quantitative data are represented as the mean ± SD of triplicate experiments. Statistical analyses were performed with the GraphPad software (InStat, Inc., San Mateo, CA) (version 3.00) using the Student *t*-test for two-group comparisons.

3. Results

3.1 *PLLA films are optimal substrates for mesoangioblast viability and growth*

Initially, we seeded mesoangioblasts on glass slides with or without 2D-PLLA films and evaluated cell morphology and growth rate within 3 days of plating. As shown in the panel of micrographs in figure 1, after 48 h of culture, PLLA did not induce any morphological change with respect to the glass substrate, indicating the ability of this material to support both adhesion and viability of the cell line under study (micrographs 1d and 1a, respectively). Moreover, addition of type I collagen to untreated and PLLA-coated glass slides (figure 1b and 1e) did not result in significant morphological changes with respect to controls. Regarding cell growth rate, as shown in figure 2, PLLA films promoted cell proliferation to a higher degree than untreated glass. In particular, while cells on untreated glass reached the plateau phase after only 2 days of culture, cells on PLLA films were still in exponential growth 3 days after plating. In parallel assays, we also tested the effect of type I collagen-coating of PLLA supports on cell viability and growth. The data obtained indicate that at 48 h, the number of cells on uncoated PLLA films was higher than that on collagen-coated PLLA supports, suggesting that collagen addition to the PLLA film does not ameliorate cell growth rate (not shown).

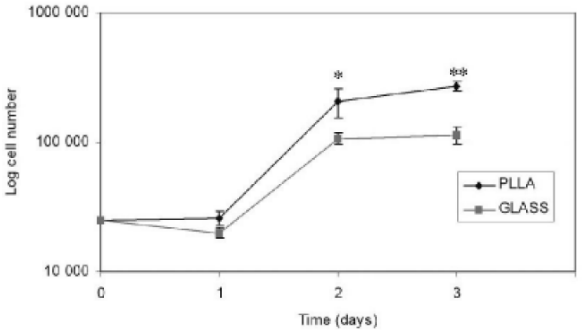

Figure 2. Proliferative behaviour of A6 cells cultured onto PLLA film (♦) or untreated glass (■). Cell number was determined by direct count after 24, 48 and 72 h of plating. The data represent the mean ± SD of three independent experiments. Significance was calculated using the Student *t*-test, and *P* values vs control (untreated glass) of 0.0005 (*) and 0.0001 (**) are indicated. The data are plotted on a semi-logarithmic scale.

3.2 *PLLA porous scaffolds allow survival and growth of inoculated mesoangioblasts*

In light of the promising results obtained using PLLA films, in a second set of experiments, we assayed whether mesoangioblasts could grow within PLLA polymeric scaffolds, produced as described by Pavia *et al.* (2008). These scaffolds possess a high level of porosity (approximately 90%), with an average pore size ranging from 40 to 50 μm, and good interconnection (interconnecting channels of about 10 μm). More details on the features of the scaffolds can be found in Pavia *et al.* (2008). Cells resuspended in culture medium at a concentration of 5×10^5 cells/ml were inoculated by applying low pressure to a syringe equipped with a needle. Cell-containing scaffolds were prepared for microscopic analyses, and cross-sections were obtained by cutting the glutaraldehyde-fixed samples with a surgical blade.

Preliminary tests were performed with different histological dyes to select those that allowed observation of cell staining without background interference by the PLLA

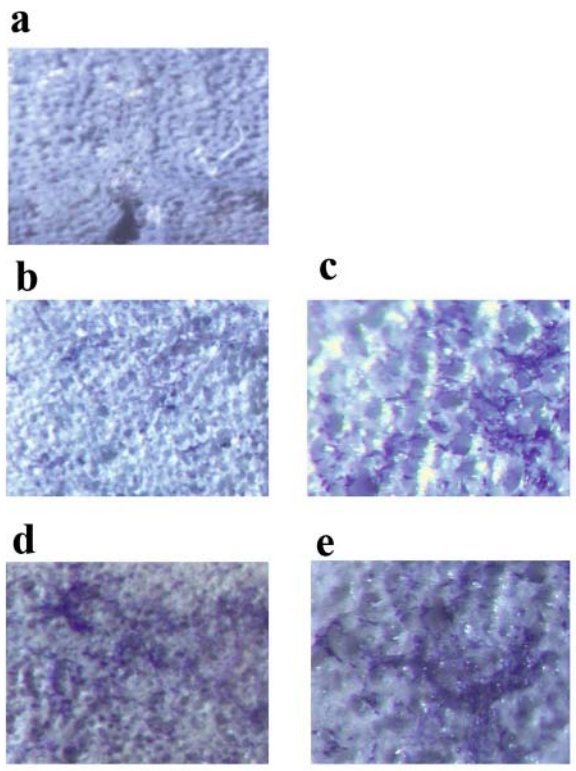

Figure 3. Toluidine blue staining of cells into PLLA scaffolds after 1 and 2 days of culture, observed under the stereomicroscope after cross-sectioning through the scaffold. **(a)** Aspect of scaffold in the absence of cells, **(b–c)** scaffold with cells observed 1 day after cell plating, **(d–e)** scaffold with cells observed 2 days after cell plating. (**a**, **b**, **d**) magnification 6.3×, (**c**, **e**) magnification 40×.

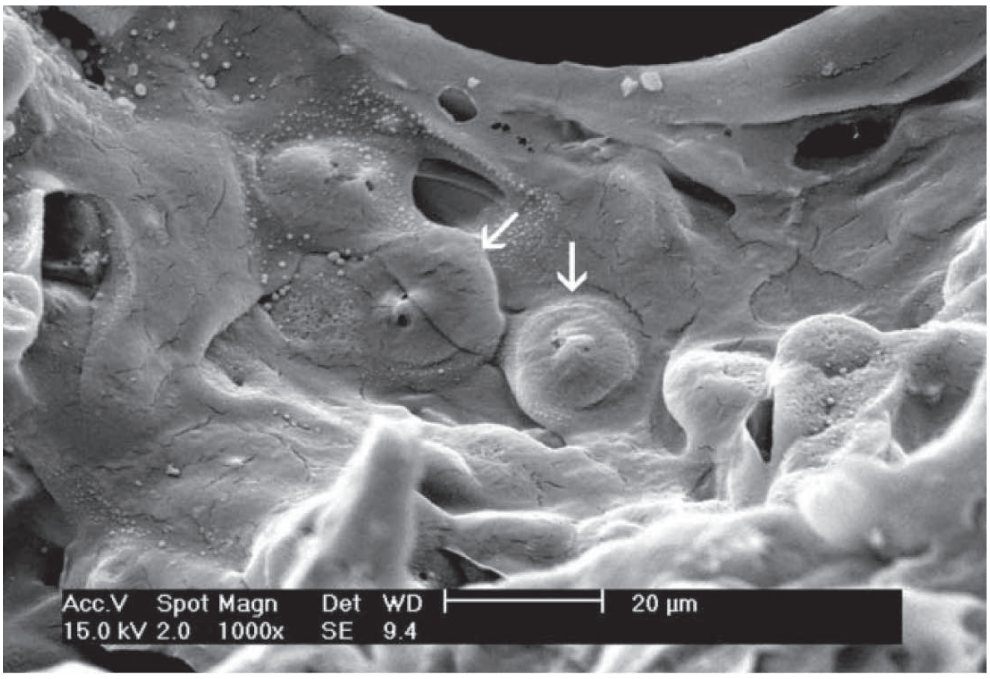

Figure 4. SEM micrograph of PLLA scaffold observed 2 days after cell plating. Arrows indicate the cells.

support. The best results were obtained using toluidine blue (not shown), which was therefore chosen for subsequent morphological analyses. Cells were then observed under a stereomicroscope after 2 days of incubation.

As shown in figure 3b, at 24 h post-inoculation, the scaffold was colonized by cells that appeared well-adhered to the internal surfaces of the pores of the polymer. At higher magnification (figure 3c), cells appeared well spread on the foam surface, as expected for live and healthy cells. After 48 h post-inoculation, the scaffold was colonized more than after 24 h (figure 3d and 3e for higher magnification). No significant differences in cell distribution were seen among all the sections observed.

These preliminary assays indicated an expansion of A6 cell colonies after several days since inoculation within PLLA polymers. This result was expected in light of the growth characteristics of the cells, shown in figure 2. Parallel morphological analyses were performed under the SEM (figure 4), which confirmed that mesoangioblasts were well attached and had spread onto the PLLA surface within the polymer pores. Detailed indications of cell proliferative capabilities and cell–polymer interactions after long-term culture will require further study.

3.3 *PLLA polymeric scaffolds are compatible with immunofluorescence assays*

A likely difficulty with the use of PLLA scaffolds for cell culture may be the inability to perform immunofluorescence assays. In fact, the expression of a differentiated phenotype, including that of committed stem cells, is commonly assessed by evaluation of the presence of specific markers detected via immunolocalization. Therefore, we checked whether cells grown within the 3D-PLLA matrix could be analysed under a confocal fluorescence microscope. In particular, to test this possibility, we performed immunofluorescence assays choosing anti-70 kDa heat shock protein (Hsp70) antibodies as a trial antibody. Hsp70 is a member of the Hsp family and we have previously demonstrated that it is constitutively and ubiquitously expressed in A6 stem cells (Geraci *et al.* 2006). As shown in the panel in figure 5, PLLA scaffolds displayed negligible intrinsic fluorescence (figure 5a), allowing the identification and detailed visualization of A6 cells grown within the 3D-PLLA matrix after 24 h (figure 5b) and 48 h (figure 5c) which, as expected, were positive to Hsp70 staining. To our knowledge, this is the first indication that confocal microscopic analysis can be performed on cells adherent to 3D-PLLA scaffolds, thereby further supporting the supposition that PLLA is a suitable substrate for the study of the differentiation pathways of stem cells in culture.

4. Discussion

In recent years, tissue engineering has benefited from the advantages offered by different polymeric foams, which have well-defined chemicophysical properties such as porosity, surface characteristics, elasticity and internal cohesiveness, in order to develop scaffolds that allow the

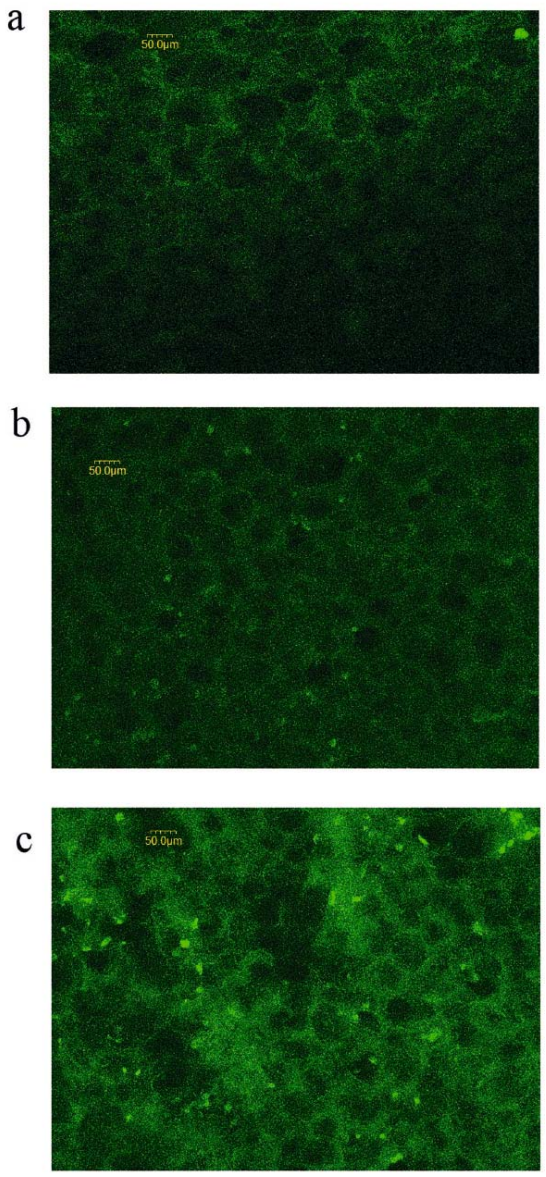

Figure 5. Hsp70 immunolocalization. **(a)** Scaffold without cells (control). **(b)** Scaffold with cells 1 day after seeding. **(c)** Scaffold with cells 2 days after seeding.

adhesion and differentiation of live cells. One of the most studied materials is PLLA which, in the present study, was used as a scaffold to evaluate the capacity of PLLA to sustain adhesion and viability of mouse A6 mesoangioblasts. The scaffolds were prepared according to a previously published technique (Pavia *et al.* 2008), allowing the formation of foams with a hierarchically ordered multilevel structure and average macropore size ranging from 10 to 100 μm. Among the variety of possible structures, we chose those foams exhibiting about 90% porosity, a pore size ranging from 40 to 50 μm, and an interconnecting channel size of approximately10 μm. The data obtained indicate that the foam which exhibits the above characteristics permits cell adhesion when used both as a 2D and as a 3D support, and that collagen coating, which is often required for cell attachment, is not needed to promote the adhesion rate of A6 cells.

Interest in the present results is two-fold: we describe here both a simple protocol allowing cell inoculation within foam pores with a very high level of cell survival, as shown by toluidine blue staining of cross-sectioned scaffolds, and a method of performing cytological analyses on cells plated within the scaffold. In the latter case, stem cells, as well as other cell types, can be induced to differentiate within the scaffolds and their pattern of differentiation can be followed by analysing the expression of specific markers. To our knowledge, this is the first time that cells within scaffolds have been observed under a confocal fluorescence microscope, allowing analysis of the constituents of specific intracellular compartments.

In conclusion, we highlight the suitability of the material used and the techniques applied for future development of these applications in the field of tissue engineering and the study of stem cell differentiation.

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