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RESEARCH ARTICLE

Industrial approach in developing an advanced therapy product for bone repair

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Abstract

Mesenchymal stem cells (MSCs) are multipotent cells with therapeutic applications. The aim of our work was to develop an advanced therapy product for bone repair, associating autologous human adipose-derived MSCs (ASCs) with human bone allograft (TBF; Phoenix®). We drew up specifications that studied: (a) the influence of tissue collection procedures (elective liposuction or non-invasive resection) and patient age on cell number and function; (b) monolayer cell culture conditions and osteodifferentiation and particularly the possibility of reducing stages of culture; and (c) the bone construct preparation and especially the comparison between two types of cells seeded on bone allograft (number of cultured processed lipoaspirate (PLA) cells and monolayer-expanded ASCs) and cultured for 1, 2 and 3 weeks. The results showed that tissue harvesting techniques and patient age did not affect PLA cell number and ASC cloning efficiency. PLA cells can be directly osteodifferentiated (instead of culturing them in expansion medium first and then differentiating them) and these cells were able to mineralize when they were cultured in an osteogenic medium containing calcium chloride. PLA cells directly seeded on bone allograft for a minimum of 3 weeks of culture in this osteogenic medium expressed osteocalcin and colonized the matrix better than monolayer-expanded ASCs. This work detailed the specifications of a pharmaceutical laboratory to develop an advanced therapy product and this current approach is promising for bone repair.

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1. Introduction

A new medical field known as regenerative medicine is developing and attracting increasing numbers of researchers, practitioners and actors in industry. Mesenchymal stem cells (MSCs), which are very good candidates for regenerative medicine, were initially discovered in bone marrow and then also found in high numbers in fat tissue (Bruder *et al*., 1994, Friedenstein *et al*., 1976, Zuk *et al*., 2001). Due to limitations in autologous human bone marrow procurement (no more than 40 ml), adipose tissue could easily be used for regenerative medicinal application (a liposuction procedure is less invasive than bone marrow aspiration) (Gimble *et al*., 2007, Mizuno, 2009, Strem *et al*., 2005).

In France, several clinical trials have been carried out in order to highlight the effectiveness of MSCs mainly in osseous repair and also during haematopoietic stem cell graft or cardiac repair after infarction (Bourin and Gadelorge, 2007). However, no advanced therapy product (using processed cells) is yet on the market.

Osseous defects of the human skeleton are common in reconstructive orthopaedic and trauma surgery.

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Autograft remains the gold standard for bone graft transplantation. Size limitation, donor site morbidity and other complications significantly limit its use for the repair of large structural defects. Allogeneic structural bone graft is one of the popular choices for clinicians, due to its osteoconductive nature, unlimited size and the absence of donor site morbidity. However, because of the lack of viable cells on processed bone allografts, healing and incorporation of the grafts in large defects are difficult (Burchardt, 1983, 1987). Thus, an alternative exists: culturing MSCs on bone allograft to bring osteogenic properties to the matrix (Bolland *et al*., 2006, Rust *et al*., 2007, Xie *et al*., 2007, Zhang *et al*., 2008).

The human cancellous bone allograft (TBF; Phoenix®) is viro-inactivated, lyophilized, γ-sterilized and usually used in orthopaedic or dental surgery to fill small bone defects. It has also been tested in association with autologous bone marrow (and not with processed cells) for clinical use (Schmid *et al*., 2007). The aim of the present study was to develop an advanced therapy product for non-union, large bone defects (excision of tumours, loss of traumatic bone substances) and diseases such as osteonecrosis. Osteogenic potential will be achieved through autologous adipose-derived stem cells (ASCs).

Based on the overview of the challenges and obstacles for engineering bone (Logeart-Avramoglou *et al*., 2005) and on our experience in development and production of Cartipatch® (an autologous chondrocyte implant) (Selmi *et al*., 2008), our specifications are as follows:

1. *Cell procurement.* Minimally invasive harvesting procedure? Influence of age? For this we compared cell characteristics between: (a) two types of harvesting, classical elective liposuction (under general anaesthetic, usually used for massive liposuction) and non-invasive resection (under local anaesthetic occasionally performed for local adipose tissue resection); and (b) two groups of women donors, one with patients aged 19–40 years and the other aged 41–67 years.
2. *Monolayer cell culture and osteodifferentiation.* Cell selection, expansion and then osteodifferentiation or direct cell osteodifferentiation (to reduce the number of stages of culture)? We compared a direct culture of processed lipoaspirate (PLA) cells in osteogenic medium to a classical culture of PLA cells in an expansion medium (named control medium or CM) usually used for MSC selection and expansion (Gindraux *et al*., 2007, Inoue *et al*., 2005, Martin *et al*., 1997). Two osteogenic media, adapted from Zuk's osteogenic medium (Zuk *et al*., 2001), were tested, one of which contained calcium chloride, the other which did not (named OM1 and OM2, respectively).
3. *Bone construct preparation.* Cell expansion in monolayer before seeding on to matrix or direct seeding on to matrix? Period of culture? For this, we tested: (a) two types of cells for the culture on the bone allograft, PLA cells that had never been cultured and monolayer-expanded ASCs; and (b) three periods of culture, 1, 2 and 3 weeks.

These findings will allow us to develop an innovative cell therapy product for bone repair and will enhance the ranges of the Phoenix product.

2. Materials and methods

2.1. Adipose tissue sampling and culture of ASCs

After obtaining informed consent, adipose tissue was recovered from women undergoing liposuction. Two clinical procedures were used to collect subcutaneous adipose tissue from women (age 19–67 years; average 44 years; $n = 17$).

2.1.1. Classical elective liposuction under general anaesthetic

In this method the tissue collected specimens corresponded to 'diluted adipose tissue'. A mix of saline solution (NaCl 0.9%) and vasoconstriction solution (adrenaline 1 mg/l) was infused into the adipose specificity anatomical location (abomen, hips, buttocks) to separate adipose tissue and to minimize blood loss. The liposuction was then continued as previously described by Zuk *et al*. (2001). In this procedure, 1.2 l diluted adipose tissue was collected from each woman.

2.1.2. Non-invasive resection under local anaesthetic

In this method the tissue collected specimens corresponded to 'concentrated adipose tissue'. For this, 60 ml concentrated adipose tissue located in the abdomen, hips and buttocks was directly drawn up with a syringe without injecting solutions.

2.1.3. Cells extraction

Diluted and concentrated lipoaspirates were processed as previously described by Zuk *et al*. and the resulting cell population was termed PLA (Zuk *et al*., 2001). Viable mononuclear cells (MNCs) contained in this PLA fraction were counted using a haematocytometer and the results were expressed by the number of MNCs/ml adipose tissue.

PLA cells were then seeded at passage 0 in a culture flask at 37 °C and 5% CO_2. The day after seeding, adherent cells corresponding to ASCs were washed with Hank's buffer (to remove residual non-adherent red blood cells) and were maintained at 37 °C and 5% CO_2.

2.2. *In vitro* ASCs; functional and phenotypic studies

Three experiments were simultaneously performed with PLA cells:

1. *Cloning efficiency.* 200 MNCs/cm^2 were seeded in CM, OM1 and OM2 (Table 1). Cloning efficiency

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Table 1. Composition of cell culture medium

Medium	Media	Serum	Supplementation
Control medium (CM)	DMEM/Ham's F12	CBS (10%)	1 ng/ml FGF2 (ref 354060, BD Biosciences) 50 mg/l antibiotics/2.5 mg/l antimycotic
Osteogenic medium 1 (OM1)	DMEM/Ham's F12	CBS (10%)	1 ng/ml FGF2 50 mg/l antibiotics/2.5 mg/l antimycotic 284 mM ascorbate (AMM3421357, Roche; bought in local pharmacy) 10 mM β-glycerophosphate (G989, Sigma-Aldrich, Saint Quentin Fallavier, France) 0.1 mM dexamethasone (9903, Merck bought in local pharmacy) 0.01 mM vitamin D3 (C1357, Sigma-Aldrich) 5 mM $CaCl_2$ (AMM3195470, Aguettant Laboratory, Lyon, France)
Osteogenic medium 2 (OM2)	DMEM/Ham's F12	CBS (10%)	1 ng/ml FGF2 50 mg/l antibiotics/2.5 mg/l antimycotic 284 mM ascorbate 10 mM β-glycerophosphate 0.1 mM dexamethasone 0.01 mM vitamin D3

was evaluated by counting the colony-forming unit fibroblastic cells (CFU-f) after 9 days of culture and expressed by the number of CFU-fs for 10^5 MNCs initially seeded.

2. *Expansion potential and flow cytometry studies.* 10×10^3 MNCs/cm^2 were seeded in CM, OM1 and OM2, cultured until confluence (i.e. for 14 days) and then trypsinized with 0.05% trypsin–ethylenediamine tetra-acetic acid (EDTA; Invitrogen, Cergy Pontoise, France). The expansion factor was determined as the ratio between the number of trypsinized MNCs and the number of MNCs initially seeded. Trypsinized ASCs were characterized by flow cytometry (EPICS XL Cytometer, Beckman Coulter, Marseille, France) and CellQuest software (BD Biosciences, Meylan, France). The cells were incubated for 30 min with mouse phycoerythrin (PE)-conjugated monoclonal antibody (mAb) anti-human CD31 (clone 1F11; Beckman Coulter), anti-human CD73 (clone AD2; BD Biosciences, Pont de Claix, France), anti-human CD105 (clone SN6, Dakocytomation, Trappes, France) and mouse fluorescein isothiocyanate (FITC)-conjugated mAb anti-human CD90 (clone 5E10, Dakocytomation) or with appropriate isotype-matched control mAb (Dakocytomation). Ten thousand events for each sample were recorded.
3. *Osteogenic differentiation.* 10×10^3 MNC/cm^2 were seeded in OM1 and OM2 and cultured for 3 weeks. Culture time was chosen in accordance with a cell therapy protocol. The presence of calcium-containing depots in the extracellular matrix of cells was visualized by von Kossa staining, as previously described (Dennis *et al*., 2002), and the detection of osteocalcin was performed using an anti-osteocalcin immunohistochemistry (mouse mAb anti-human osteocalcin, clone 6F9G4E10; AbD Serotec, Dusseldorf, Germany). This phenotype control was validated by an external company, Novotec, as for the procedure involved for Cartipatch. Positive control was achieved using an osteoblastic-lineage MG-63 maintained in OM 2; the cell line was isolated from a human osteosarcoma (American Type Culture Collection, Rockville, MD, USA).

2.3. Bone construct preparation and characterization

The bone allograft Phoenix used in this study corresponded to cubes of approximately $6 \times 6 \times 6$ mm (reference B04: www.tbf-lab.com). We tested two types of cells to analyse the possibility of reducing the culture time, PLA cells (directly seeded on the bone allograft) and 'monolayer-expanded ASCs', which corresponded to PLA cells expanded at passage 0 in monolayer in CM until confluence, then trypsinized and seeded at passage 1 on bone allograft. So, PLA cells and monolayer-expanded ASC were seeded at 2×10^6 MNC/cm^3 on bone allograft and incubated in a culture flask in OM1 under gentle agitation for 1, 2 and 3 weeks. The medium was changed every 3 days. Cell density and culture time were chosen in accordance with a cell therapy protocol. Bone constructs were then fixed in 4% parafolmaldehyde, decalcified in EDTA solution, embedded in paraffin and sectioned. The cells were characterized by haematoxylin–eosin–saffran (HES) staining and osteocalcin immunohistochemistry (rabbit polyclonal antibody FL-100; SC-30044, Santa Cruz Biotechnology, distributed by Tebu-bio, Le Perray en Yvelines, France). Control immunostaining was performed on bone allograft cultured in OM1 without cells for 1 month. These histological techniques and phenotype controls were realized by Novotec. For some experiments, cells seeded on bone allograft were harvested by dissolution of the bone and flow cytometric studies were performed on these cells (as described above).

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Table 2. Relation between the adipose tissue collection techniques and patient age on viable MNCs number/ml adipose tissue and on ASC cloning efficiency (± SME)

	Adipose tissue collection techniques		Patient age		Average
	Elective liposuction ($n = 11$)	Non invasive resection ($n = 6$)	19–40 years (34 ± 4) ($n = 10$)	41–67 years (58 ± 5.4) ($n = 7$)	44 years (±13) ($n = 17$)
Viable MNCs (number/ml)	$149 \pm 53 \times 10^3$	$159 \pm 34 \times 10^3$	$143 \pm 35 \times 10^3$	$166 \pm 59 \times 10^3$	$152 \pm 49 \times 10^3$
Cloning efficiency	$4045 \pm 2192 \times 10^{-5}$	$4572 \pm 1626 \times 10^{-5}$	$4007 \pm 2008 \times 10^{-5}$	$5220 \pm 1782 \times 10^{-5}$	$4338 \pm 1789 \times 10^{-5}$

2.4. Statistical analysis

Numerical values were reported as means ± SEM. Shapinro–Wilk tests showed that we could not reject the data normality hypothesis: comparisons between means were performed using Student's *t*-test. The difference was statistically significant when $p < 0.05$.

3. Results

3.1. Collection procedure and patient age influences?

For each patient, the viable MNCs in the PLA fraction were counted and the results are presented in Table 2. The results showed that the two harvesting techniques allowed a similar number of cells to be collected: the average number was $149 \pm 53 \times 10^3$ MNCs/ml ($n = 11$) for adipose tissue collected by elective liposuction procedure and was $159 \pm 34 \times 10^3$ MNCs/ml ($n = 6$) for non-invasive resection ($p < 0.05$). The ASCs were then cultured in CM and CFU-f were counted after 9 days of culture (Table 2). Cloning efficiency results showed no difference between elective liposuction ($4045 \pm 219 \times 210^{-5}$) and non-invasive resection ($4572 \pm 1626 \times 10^{-5}$), confirming that the two collection procedures were comparable ($p < 0.05$).

The number of viable MNCs in the PLA fraction was independent of donor age (Table 2): the average number was $143 \pm 35 \times 10^3$ MNCs/ml for women aged 19–40 years (average 34 ± 4 years, $n = 10$) and was $166 \pm 59 \times 10^3$ MNCs/ml for women aged 41–67 years (average 58 ± 5.4 years, $n = 7$) ($p < 0.05$). Moreover, we showed in Table 2 that cloning efficiency did not depend on donor age, specifically: $4007 \pm 2008 \times 10^{-5}$ for women aged 19–40 years and $5220 \pm 1782 \times 10^{-5}$ for women aged 41–67 years ($p < 0.05$).

3.2. *In vitro* functional and phenotypic characterization

3.2.1. Cloning efficiency

Table 3 showed that cloning efficiency was $4338 \pm 1789 \times 10^{-5}$ ($n = 9$) for ASCs cultured in CM and was $678 \pm 77 \times 10^{-5}$ ($n = 9$) for ASCs cultured in OM2, showing a significant ($p < 0.05$) decrease by a factor of 6.4. CFU-f cultured in OM1 were difficult to count because the clones had started mineralization.

Table 3. Effect of CM and OM2 on ASC cloning efficiency and on expansion factor (± SME)

CM		OM2	
Cloning efficiency	Expansion factor	Cloning efficiency	Expansion factor
$4338 \pm 1789 \times 10^{-5}$* ($n = 9$)	2.94 ± 0.47 ($n = 11$)	$678 \pm 77 \times 10^{-5}$* ($n = 9$)	7.85 ± 3 ($n = 4$)

*$p < 0.05$.

3.2.2. Expansion potential

At confluence (day 14), the expansion factor was 2.94 ± 0.47 ($n = 11$) for ASCs cultured in CM and was 7.85 ± 3 ($n = 4$) for ASCs cultured in OM2, i.e. 2.7-fold lower ($p < 0.08$). As the culture of ASCs in OM1 had started mineralization after 5 days of culture, the cell multiplication was slightly slow compared to culture in OM2 and CM, and the trypsinization was not efficient (the expansion factor was not determined).

3.2.3. Osteogenic differentiation

After 3 weeks of osteodifferentiation, microscopic observations showed that the cell morphology differed according to the culture medium used: the cells had a spangle-shaped and disordered phenotype when cultured in OM1 and OM2 compared to negative control, i.e. ASCs cultured in CM (which had a fibroblast-like elongated phenotype). Von Kossa staining was positive for ASCs cultured in OM1 (Figure 1B), and negative for ASCs cultured in CM (Figure 1A) and in OM2 (same data as Figure 1A). Microscopic visual assessment showed that 10% of cells cultured in OM2 (Figure 1E, F) were positive for the osteocalcin marker and there was no significant difference from the positive control, i.e. osteoblastic-lineage cells cultured in OM2 (Figure 1G, H) and negative control, i.e. ASCs cultured in CM (Figure 1C, D). ASCs cultured in OM1 showed the same results as in Figure 1E, F.

Finally, the flow cytometric results revealed a homogeneous population (Figure 2B) composed of medium and large-sized cells with high granularity (region C)

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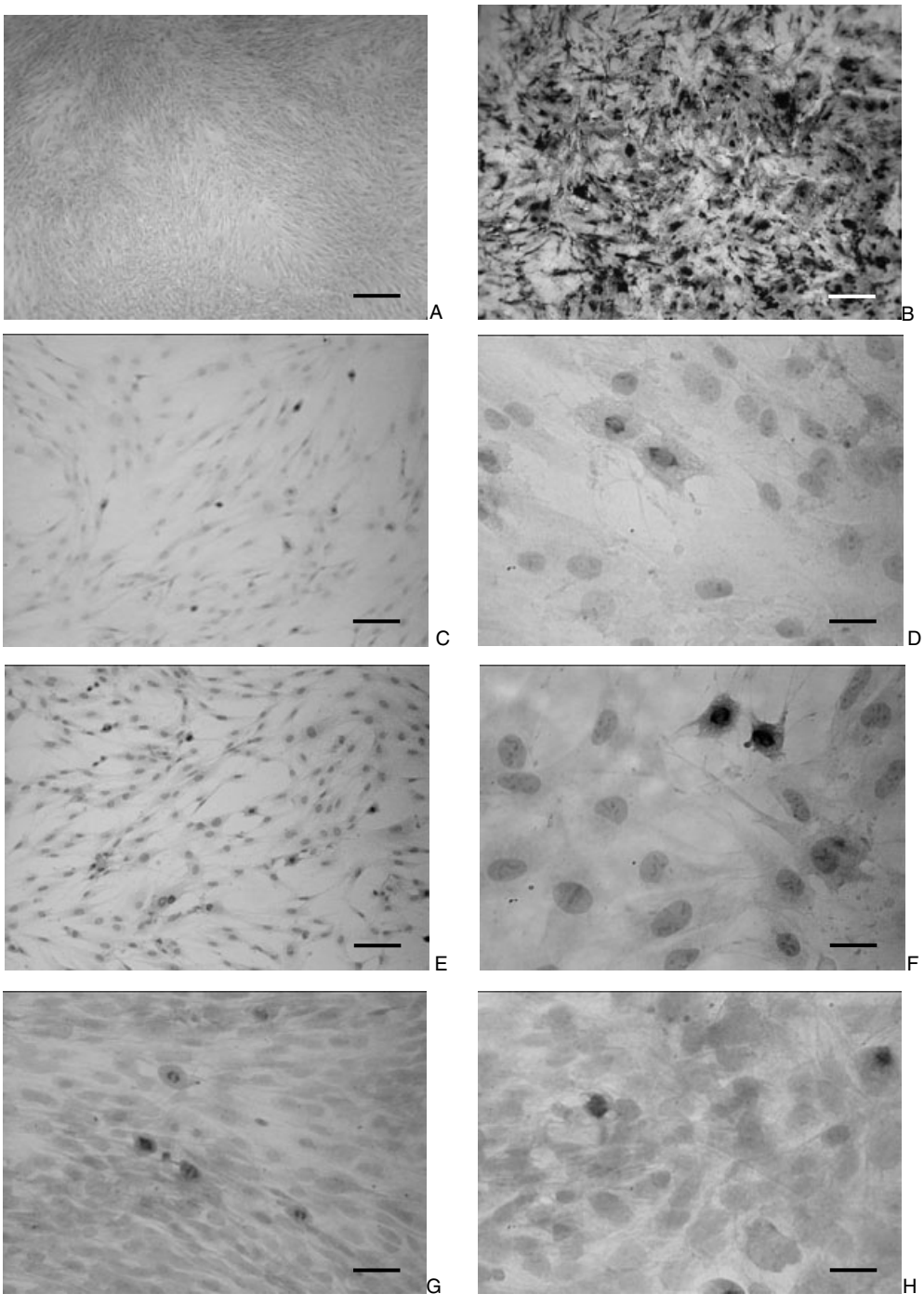

Figure 1. ASCs and osteoblastic lineage cells in *in vitro* functionality studies. Von Kossa staining of ASCs cultured for 3 weeks in CM (A) and OM1 (B). Osteocalcin detection of ASCs (C–F) and osteoblastic lineage cells (G, H) cultured for 3 weeks in CM (B–D) and in OM2 (E–H). Scale bar = 10 μm (D, F, H); 50 μm (G); 100 μm (C, E) and 200 μm (A)

for ASCs cultured in OM2, and a heterogeneous population (Figure 2A) for PLA cells composed of medium and large-sized cells with high granularity (region A) and small-sized cells and low granularity (region B) corresponding to red blood cells. The percentage of cells expressing CD31, CD73, CD90 and CD105 markers was high (90–97%) (Figure 2D, E), except the CD31 marker (3.3%) (Figure 2C) for ASCs cultured in OM2 and lower (18%, 32%, 40% and 25%, respectively) for the PLA fraction. The same percentages as those found for ASCs cultured in OM2 were observed for ASCs cultured in CM and osteoblastic-lineage cells maintained in OM2. Flow cytometry analyses of ASCs cultured in OM1 could not be carried out, due to the problem of culture trypsinization.

3.3. ASC adhesion and osteodifferentiation on bone allograft

Microscopic observations of PLA cells and monolayer-expanded ASCs cultured at 2×10^6 MNCs/cm^3 on bone

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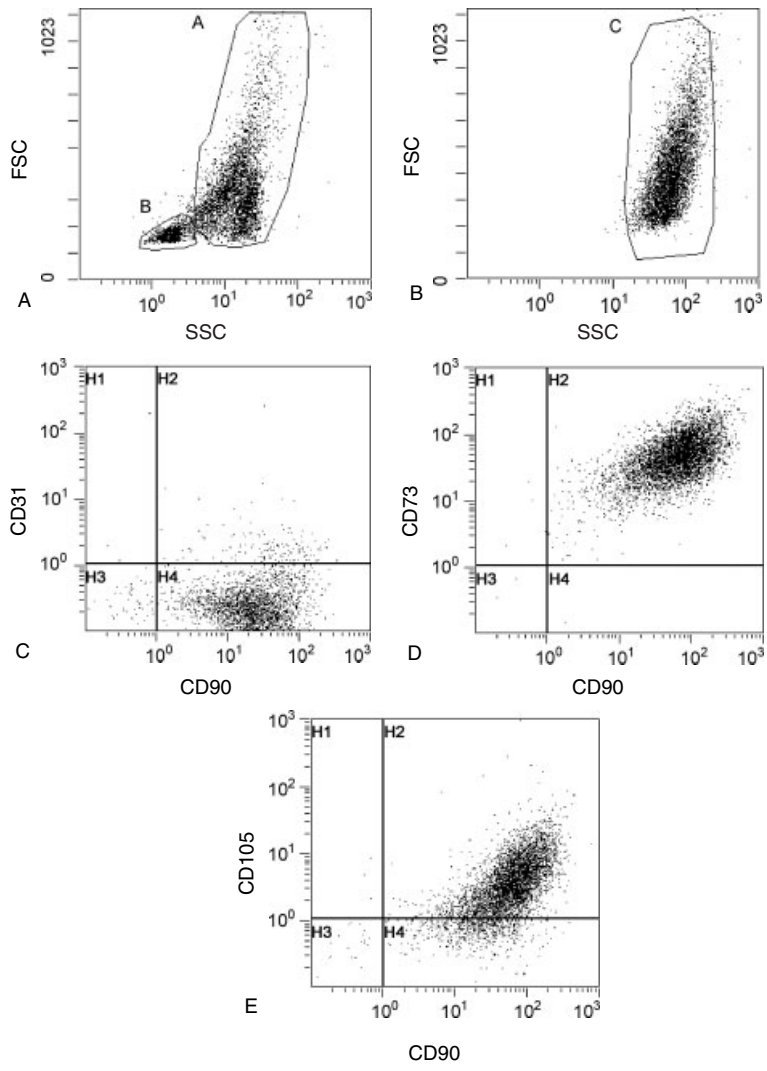

Figure 2. Flow cytometric characterization of PLA cells (A) and ASCs cultured for 3 weeks in OM2 (B–E). PLA cells (A) represented a heterogeneous population composed of medium- to large-sized cells with high granularity (region A) and small-sized cells with low granularity (region B), corresponding to red blood cells. ASCs (B) corresponded to a homogeneous population composed of medium- to large-sized cells with high granularity (region C), which expressed simultaneously CD90/CD73 (D) and CD90/CD105 (E) markers and did not express CD31 (C)

allograft for1, 2 and 3 weeks in OM1 showed that the cells were grafted essentially onto the bone allograft rather than on the culture flask.

The histological results are shown in Figure 3. At day 7, microscopic visual assessment of positive cells for osteocalcin marker (black star) was 30–40% for PLA cells (Figure 3A) and 20–30% for monolayer-expanded ASCs (Figure 3B); at day 14, 40–50% for PLA cells (Figure 3C, E) and 30–40% for monolayer-expanded ASCs (Figure 3D, F); at day 21, 50–60% for PLA cells (Figure 3G, I) and 40–50% for monolayer-expanded ASCs (Figure 3J, L). At day 21, microscopic observations showed that PLA cells (Figure 3I) colonized better bone allograft than monolayer-expanded ASCs (Figure 3J). Figure 3H and K shows the presence of osteoblasts along the bone allograft (black arrow). Immunohistochemistry performed on bone allograft cultured alone in OM1 for 1 month showed no osteocalcin detection (Figure 3M).

Flow cytometic studies performed on PLA cells and monolayer-expanded ASCs seeded on to bone allograft for 1 week in OM1 showed similar results to those seen in Figure 2B–D.

4. Discussion

The first parameter observed in this study was cell procurement and particularly the minimally invasive harvesting procedure and the influence of age. Some authors reported that resection and tumescent liposuction seem to be preferable to ultrasound-assisted liposuction for tissue-engineering purposes (Oedayrajsingh-Varma *et al*., 2006). Others observed that 24 h overnight storage leads to a significant loss of preadipocytes in excised tissue but not in the liposuction material (von Heimburg *et al*., 2004). Thus, we compared two types of procedure: classical elective liposuction under general anaesthetic [the volume collected (1.2 l) corresponded to 'diluted

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adipose tissue'] and the non-invasive resection under local anaesthetic [the volume collected (60 ml) represented 'concentrated adipose tissue']. Our results have shown that neither elective liposuction nor invasive resection affected the viable cell number in the PLA fraction or ASC cloning efficiency, contrary to the results of others (Oedayrajsingh-Varma *et al*., 2006, von Heimburg *et al*., 2004). Thus, these two types of procedure can be used in our cell therapy protocol, with a preference for non-invasive resection because it was less disruptive (we note that non-invasive resection can easily obtain 200 ml 'concentrated adipose tissue') (Mizuno, 2009). Concerning the influence of patient age, we analysed adipose tissue from two groups of women donors: one with patients aged 19–40 years and the other 41–67 years (we did not have access to the adipose tissue

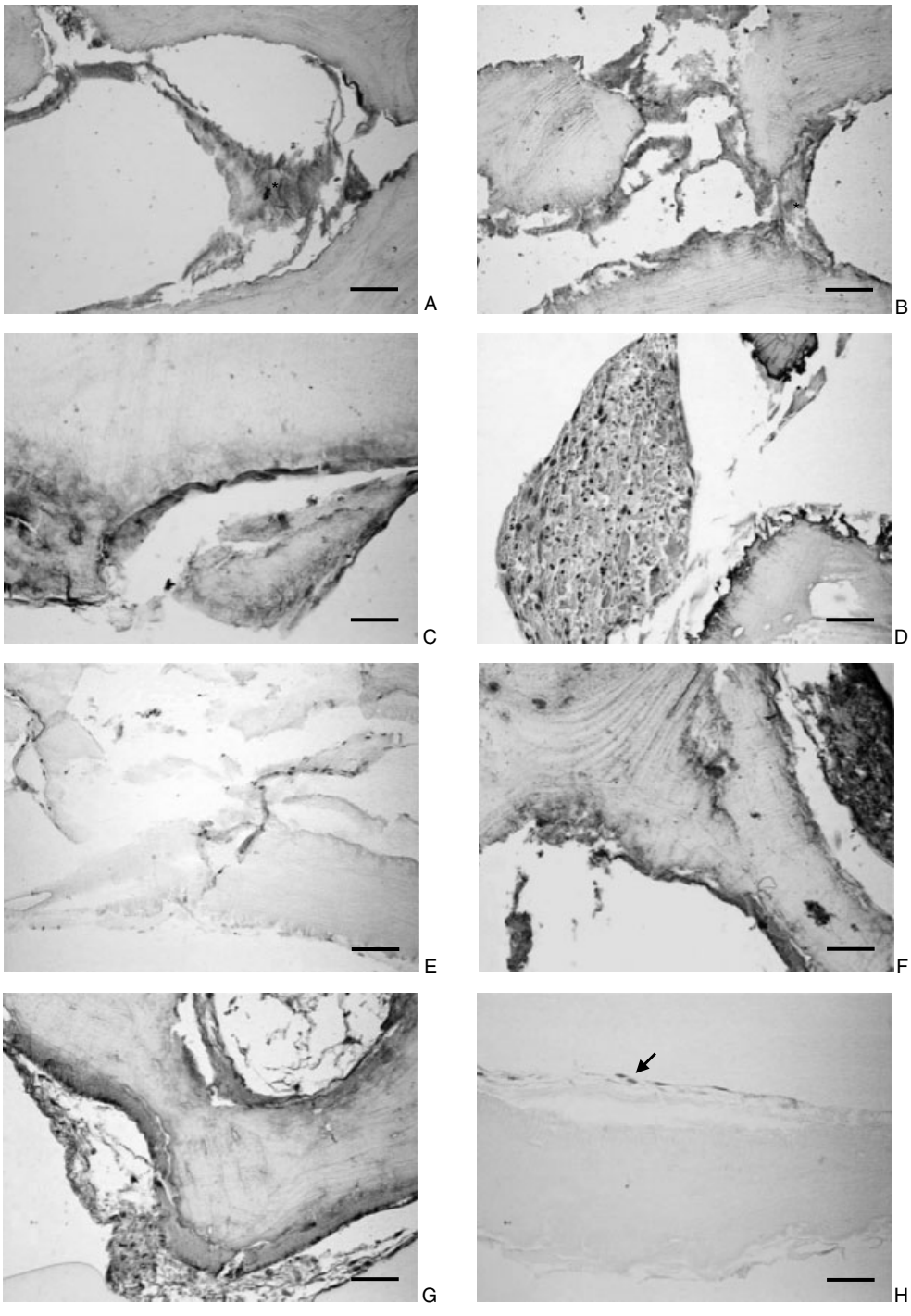

Figure 3. PLA cells and monolayer-expanded ASCs seeded on bone allograft. Cells were cultured at 2×10^6 cells/cm^3 of bone allograft in OM1 for 1 (A, B), 2 (C–F, H) and 3 weeks (G, I–L). The right column corresponds to PLA cells and the left column corresponds to monolayer-expanded ASCs. Control was achieved by culturing bone allograft alone for 1 month in OM1 (M). (A–C, F, G, I, J, L, M) Anti-osteocalcin immunohistochemistry; (D) HES staining; (E, H, K) control of immunostaining. Star, osteocalcin matrix; black arrow, osteoblasts. Scale bar = 50 μm (A, B, E, J) and 100 μm (C, D, F–I, K–N)

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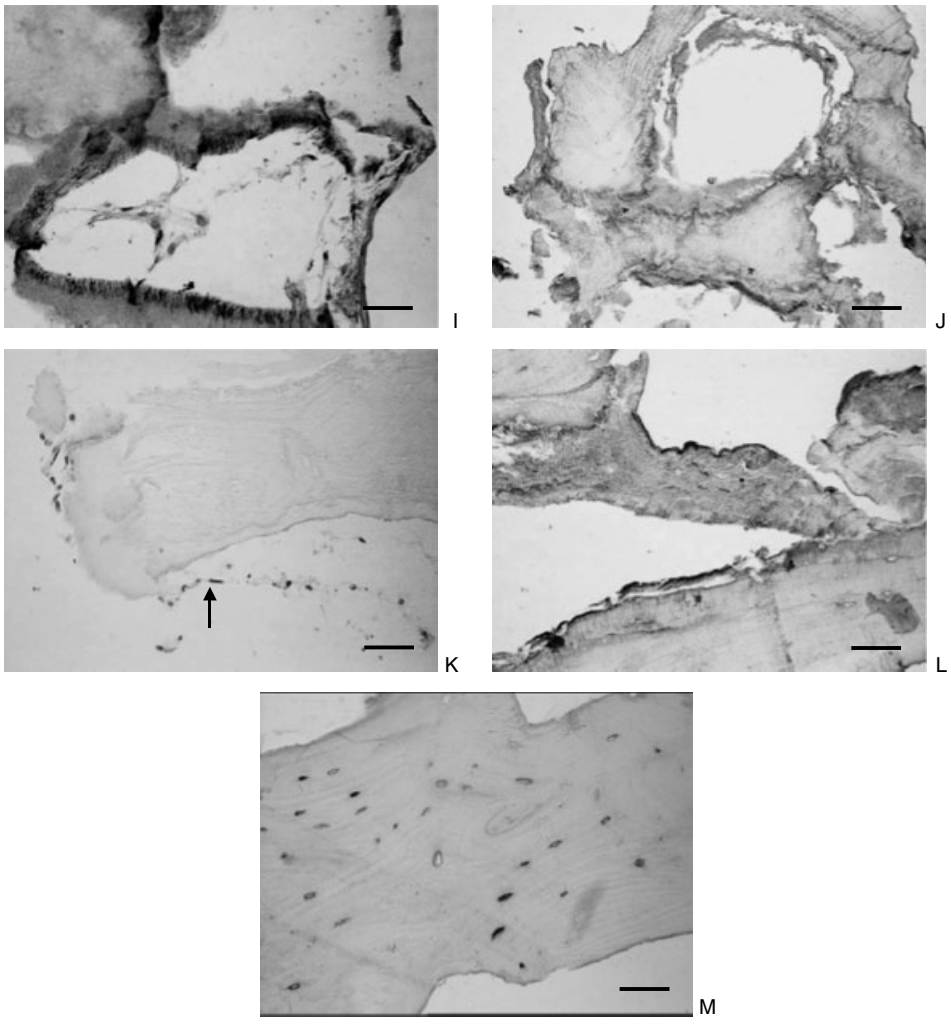

Figure 3. (*Continued*)

of patients aged >67). Our results confirmed data also reported by others (Aust *et al.*, 2004) and showed that, contrary to bone marrow MSCs (D'Ippolito *et al.*, 1999), the number of viable MNCs in the PLA fraction and ASCs cloning efficiency did not vary with patient age. This was a very important parameter because, in a cell therapy protocol for bone repair, patients may be elderly.

These first results showed that independently of the harvesting technique and patient age (average 44 ± 13 years), the average number of viable MNCs in the PLA fraction was 152×10^3 MNCs/ml (Table 2), which was similar to those reported by other authors (Mitchell *et al.*, 2006) and the ASC cloning efficiency average was $4338 \pm 1789 \times 10^{-5}$ (Table 2). We concluded that 4.3% of PLA cells were clonogenic and one CFU-f was found in approximately 23 PLA cells, results similar to those reported by other authors (Mitchell *et al.*, 2006). So, 200 ml adipose tissue will contain 30.4×10^6 MNCs, which is more than can be contained in 40 ml bone marrow (Mizuno, 2009; Strem *et al.*, 2005). As the ASC expansion factor was 2.94 (Table 3), 200 ml human adipose tissue will contain approximately 90×10^6 ASCs after 14 days of culture in CM, whereas 40 ml bone marrow contains 2.4×10^4 MSCs (Strem *et al.*, 2005).

The second parameter examined in this study consisted in evaluating the possibility of directly osteodifferentiating the PLA cells, by culturing them in an osteogenic medium instead of culturing them in a classical medium, to allow ASC selection and expansion to occur, and then osteodifferentiating them. This question was crucial because, in a cell therapy protocol, the time necessary to produce the implant is very important and the period of culture did not exceed 3 weeks. We compared cell function and phenotype according to the medium (Table 1): a CM described for MSC selection (Gindraux *et al.*, 2007, Inoue *et al.*, 2005, Martin *et al.*, 1997) and two osteogenic media (OM1 and OM2) adapted from Zuk *et al.* (2001). When ASCs were cultured in OM2, they reached confluence faster and their expansion factor was 2.7 times better than cells cultured in CM, but their cloning efficiency was significantly decreased. This finding was explained by microscopic observations, which showed that the CFU-f cultured in OM2 were composed of more cells than the CFU-f cultured in CM, probably due to the synergy of dexamethasone and fibroblast growth factor 2 (FGF2) (Lee *et al.*, 2009, Scutt and Bertram, 1999). Concerning OM1, the expansion factor was not determined because the cells could not

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be trypsinized. Flow cytometric studies performed on ASCs cultured in OM2 (Figure 2C–E) revealed the same phenotype as ASCs cultured in CM, i.e. strongly expressing CD73, CD90 and CD105 markers and not expressing CD31 marker (Aust *et al*., 2004, Dicker *et al*., 2005, Lee RH, 2004, Meyerrose *et al*., 2007, Mitchell *et al*., 2006). This mesenchymal origin was also found for the osteoblastic lineage cells maintained in OM2, and similar results were reported by Park *et al*. (2007) for cultured human periosteal-derived cells. Concerning the osteodifferentiation potential, von Kossa staining was positive for ASCs cultured in OM1 (Figure 1B) and negative for ASCs cultured in OM2 and CM (Figure 1A). The difference between the two osteogenic medium was due to the presence of calcium chloride in OM1, which allows the mineralization of cells and does not allow trypsinization to take place [Takagishi *et al*. (2006) observe that Ca^{2+} allows osteogenesis to be promoted by MG63 osteoblast-like cells). In our experiments, von Kossa staining was specific to synthesized mineralized matrix and not due to the precipitation of the calcium chloride content in OM1, because the staining was specifically localized on CFU-f. These data led us to verify the osteoblastic phenotype of ASCs cultured in OM2 by osteocalcin immunostaining. The results showed no significant difference between ASCs cultured in CM (Figure 1C, D), in OM1 and in OM2 (Figure 1E, F) and osteoblastic-lineage cells (Figure 1G, H). Expression of osteocalcin by ASCs cultured at passage 0 in CM was surprising and could be explained by the spontaneous differentiation of MSCs in the osteoblastic lineage, as also found by Banfi *et al*. (2000) for bone marrow MSCs; the intermediate level of osteocalcin detection in osteoblastic-lineage cell cultures may be due to the fact that human osteosarcoma MG63 cells have a phenotype of relatively immature osteoblasts (Takagishi *et al*., 2006). A time of culture exceeding 3 weeks could enable us to observe a significant difference.

These second data led us to respond to the above question, i.e. that PLA cells can be directly osteodifferentiated: cultured in OM1, they were able to mineralize; cultured in OM2, they were unable to mineralize but they greatly proliferated. The fact that OM2 did not allow mineralization to occur was surprising in view of the literature (Im *et al*., 2005, Zhou *et al*., 2006). De Girolamo *et al*. (2007) observe the same results with one of their osteogenic media.

The third parameter consisted of developing the advanced therapy product. As OM1 allowed mineralization to occur, we decided to use this medium for preparing bone constructs. We tested two types of cells for culture on bone allograft (PLA cells and monolayer-expanded ASC in CM) and different periods of culture (1, 2 and 3 weeks); CM was chosen for the ASC monolayer culture because it remains the 'gold standard' and a validated medium for ASC selection and expansion. Microscopic observations showed that cells were grafted on bone allograft rather than on the culture flask. This demonstrated that Phoenix was favourable for cell adhesion. The histological results showed that PLA cells produced slightly more osteocalcin (with a peak after 3 weeks of culture) and colonized better bone allograft than monolayer-expanded ASCs (Figure 3). This last finding can be explained by the fact that PLA cells have never been cultured and would probably present a different graft with bone allograft than monolayer-expanded ASCs. This hypothesis can also be explained by the presence of non-adherent mesenchymal progenitors (NAMPs) (Wan *et al*., 2006) in only the supernatant of PLA culture (and not in the supernatant of monolayer-expanded ASCs) which gradually binds on to bone allograft. NAMPs are a class of multipotent cells that can generate an adherent progeny and their progeny showed an increased proliferation capacity and a greater differentiation potential than that of the initially adherent CFU-f (results to be published soon by I Martin *et al*.).

Flow cytometric studies performed on PLA cells and monolayer-expanded ASCs cultured on bone allograft in OM1 showed a MSC phenotype, i.e. negative for the CD31 marker and simultaneously positive for CD73/CD90 and CD105/CD90 markers (same results as Figure 2B). These final data led us to conclude that culture on bone allograft in OM1 allowed ASC selection as a classical culture in monolayer on a plastic flask and validate the possibility of culturing PLA cells directly onto bone allograft in osteogenic medium, to reduce the number of different stages of the protocol.

Other authors have also investigated the culture of MSCs (or monolayer-expanded ASCs) on bone allograft in animals (Donati *et al*., 2008, Eniwumide *et al*., 2007, Follmar *et al*., 2007, Zhang *et al*., 2005) or in clinical studies (Bolland *et al*., 2006). This paper describes for the first time the use of a French-manufactured bone allograft in a cell therapy protocol.

This study allowed us to respond to our specifications:

1. *Cell procurement.* Non-invasive resection under local anaesthetic can be used to reduce the harvesting procedure and patient age did not influence cell number and functionality (cloning efficiency).
2. *Cell culture.* ASCs can be directly cultured in osteogenic medium to reduce the stages of culture. We note that if cells do not need to be trypsinized, the preference is for OM1, which allows mineralization of ASCs to occur.
3. *Bone construct preparation.* The ideal combination seems to be the seeding of PLA cells directly onto bone allograft for a minimum of 3 weeks of culture in OM1.

After having demonstrated the presence of ASCs capable of mineralization in rabbit and sheep adipose tissue, we adapted our cell therapy protocol to these animal models by producing rabbit and sheep bone allografts (using the same procedure as for Phoenix). Bone repair models (a calvarial defect in rabbits and a metatarsus defect in sheep) filled with bone allograft containing autologous ASC are under investigation.

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This study gives details of the specifications of a pharmaceutical laboratory to develop an advanced therapy product using ASCs and a commercialized bone allograft. This product will complete the other cell and tissular therapy products also developed and commercialized by TBF.

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