¹¹¹Indium-oxine labelling for evaluating the homing process of autologous osteoblasts implanted percutaneously in atrophic nonunion fractures

Jean-Philippe Hauzeur · Claire Bernard ·
Dominique Egrise · William Kurth ·
Henry Van Cauwenberge · Chantal Lechanteur ·
Philippe Gillet · Yves Beguin · Michel Malaise ·
Roland Hustinx

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Abstract

Purpose The aim of the study was to control the in vivo localisation of implanted cells in cell-based therapies. Labelling cells with ¹¹¹indium-oxine is one of the most interesting methods proposed. We evaluated this method in the setting of autologous osteoblast implantation in nonunion fractures.

Methods An in vitro study of osteoblasts was conducted after ¹¹¹indium-oxine labelling. Radioactivity retention and viability, proliferation and the ability to produce alkaline phosphatase were evaluated in a seven-day culture. In vivo

labelling of implanted osteoblastic cells was conducted during a therapeutic trial of atrophic nonunion fractures, with the leakage outside the nonunion site and local uptake evolution at four, 24 and 48 hour being studied.

Results The mean labelling efficiency for osteoprogenitors was 78.8±4.6 %. The intracellular retention was 89.4±2.1 % at three hours and 67.3±4.7 % at 18 hours. The viability assessed at three hours was 93.7±0.6 %. After seven days of culture, morphology and alkaline phosphatase staining were similar for both labelled and unlabelled control cells, although the proliferation rate was decreased in the labelled cells. Some

J. Ph. Hauzeur and C. Bernard contributed equally to this work.

J.-P. Hauzeur (✉) · M. Malaise
Department of Rheumatology, CHU Sart Tilman,
University of Liège, B35, 4000 Liège, Belgium
e-mail: jean-philippe@hauzeur.org

M. Malaise
e-mail: michel.malaise@ulg.ac.be

C. Bernard · R. Hustinx
Department of Nuclear Medicine, CHU Sart Tilman,
University of Liège, Liège, Belgium

C. Bernard
e-mail: C.Bernard@chu.ulg.ac.be

R. Hustinx
e-mail: rhustinx@chu.ulg.ac.be

D. Egrise
Department of Nuclear Medicine, Erasme University Hospital,
University of Brussels, Brussels, Belgium
e-mail: degrise@ulb.ac.be

W. Kurth · H. Van Cauwenberge · P. Gillet
Department of Orthopaedic Surgery, CHU Sart Tilman,
University of Liège, Liège, Belgium

W. Kurth
e-mail: w.kurth@chu.ulg.ac.be

H. Van Cauwenberge
e-mail: henry38@yahoo.fr

P. Gillet
e-mail: philippe.gillet@chu.ulg.ac.be

C. Lechanteur · Y. Beguin
Laboratory of Cell and Gene Therapy, CHU Sart Tilman, Liège,
Belgium

C. Lechanteur
e-mail: c.lechanteur@chu.ulg.ac.be

Y. Beguin
e-mail: Yves.beguin@chu.ulg.ac.be

local intraosseous leakage was observed in four of 17 cases. All patients showed uptake at the injection site, with four having no other uptake. Four patients showed additional uptake in the bladder, liver and spleen, while 11 patients had additional uptake in the lungs in addition to the bladder, liver and spleen. The activity ratios (injection site/body) were 48 ± 28 % at four hours, 40 ± 25 % at 24 hours and 35 ± 25 % at 48 hours. After correcting for decay, the activity within the injection site was 82 ± 15 % at 24 hours and 69 ± 11 % at 48 hours compared with the activity measured at four hours. No relationship was found between uptake and radiological bone repair.

Conclusions The ^{111}In -indium-oxine labelling appears to be a good method for monitoring the behaviour of the osteoblastic cells after their implantation in atrophic nonunion fractures.

Introduction

During recent years, a new therapeutic strategy has been developed in order to increase bone repair in osteonecrosis (ON) [1] and atrophic nonunion (NU) fractures [2]. This treatment consists of a percutaneous implantation of bone marrow stem cells (BMSC) or BMSC differentiated into osteoblasts in the lesion.

Certain questions still need to be resolved before such a therapeutic strategy may be validated. Are the cells situated in the correct place or is some leakage reducing the number of the cells active in the bone repair? Are the cells staying in the correct site until their activation allows for local adhesion and proliferation? Previous publications provided no answers to these parameters of therapy. The accuracy of the implantation and later homing of the implanted cells require further evaluation. In a previous study, the early positions of the implanted cells were studied in several cases of hip ON using the labelling of cells with ^{111}In [3]. This cell labelling technique appears interesting, as it may prove to be a safe method in human trials. Thus, we aimed to confirm the safety of BMSC labelling, especially in terms of the osteoblastic differentiation. Another aim of our study was to investigate the effectiveness of this method in quantifying the number of cells remaining in the site just after the injection and over the following days.

This study was therefore conducted in order to assess the safety of ^{111}In labelling on osteoblasts in vitro. Thereafter, ^{111}In -labelled cells were used in vivo in a trial on atrophic NU fractures treated by locally implanting autologous osteoblasts. An evaluation of leakage of the labelled cells outside the NU site, along with its effect on the final result of the bone reconstruction, was performed, in addition to the evaluation of the diffusion and the homing of these labelled cells.

Materials and methods

In vitro study

Evaluating radioactivity retention and the effects of ^{111}In labelling on cellular viability

Methods of labelling A total volume of 30 ml of bone marrow was collected from the iliac crest. Mononucleated cells were recovered from the bone marrow sample using Ficoll gradient centrifugation. The cells were washed with phosphate-buffered saline (PBS) and deposited in culture flasks with medium containing X-Vivo 20 (clinical grade) serum-free medium (Cambrex), 5 % autologous plasma and 2 ng/ml fibroblast growth factor (FGF), at a density of 10 million cells/175 cm². At day 14 the cells were recovered using Versene/ethylenediaminetetraacetic acid (EDTA) and passaged for further culture in the same medium at a density of 1 million/175 cm². One week later the cells were harvested.

For cell labelling, 2×10^6 osteoblast cells were suspended in 200 μl culture medium. The cell suspension was incubated with 7.4 MBq ^{111}In -indium-oxine (Tyco Healthcare, Mechelen, Belgium) for 25 min at room temperature. Following the incubation period, the cells were washed once in culture medium and then twice in PBS at 250 g for five minutes. Finally, the cells were suspended in PBS supplemented with 1 mg/ml human serum albumin.

An aliquot of the cell suspension was taken and diluted in culture medium and maintained at 37 °C. To evaluate the cellular retention of ^{111}In and effects of ^{111}In -indium-oxine labelling on cellular viability, all samples were inspected immediately after cellular labelling and at three hours and for some samples 18 hours after labelling. Cell viability was assessed using the trypan blue dye exclusion technique.

Methods of evaluation To evaluate ^{111}In cellular retention, cells and supernatants were separated by centrifugation at 250 g for five minutes. The radioactivity of cells and supernatants was measured in a Capintec CRC-12 calibrator (Canberra, Belgium). Labelling efficiency and the percentage of ^{111}In retention at three and 18 hours were expressed as the radioactivity of cells divided by the total radioactivity of cells and supernatants. Results were expressed as mean $\pm$ standard error of the mean (SEM).

For three samples, the labelled cells along with the unlabelled control cells were plated at a density of 5×10^3 cells/cm² in six-well culture plates in culture medium and incubated at 37 °C in a humidified atmosphere of 95 % air/5 % CO₂. The cells were observed with an inverted microscope every day during a week, and were then fixed and stained for alkaline phosphatase (Kit 85 L-2, Sigma-Aldrich, Bornem, Belgium) at day seven.

In vivo study

Evaluation of osteoblast homing in patients

Patients The Institutional Ethics Committee approved the study protocol. In total, 20 patients with atrophic NU fracture were prospectively recruited. The patients were part of a cohort enrolled in a trial evaluating the safety and efficacy of a cell-based treatment of NU fractures by implanting autologous osteoblasts into the NU site. All patients signed a written consent form. NU diagnosis was based on radiological evidence, with signs consisting of the absence of bone bridging between the fragments, persistent fracture lines, sclerosis at the fracture ends and absent callus [4].

The mean age was 44 years (range 18–62), and the sex ratio was four women to 16 men. The NU fracture was located in the upper limb in nine cases (one wrist, one ulna, three radii and four humeri), lower limb in seven cases (two fibulae, three tibiae and two femoral necks), clavicle in three cases and sternum in one case.

¹¹¹Indium-oxine osteoblast cell labelling Firstly, 40 ml of bone marrow was collected from the iliac crest and cultured following a three week special programme in order to obtain autologous osteoblasts. Thereafter, 10 % of the total amount of BMSC was incubated for 30 minutes with 200 μ Ci ¹¹¹indium-oxine at a concentration of 1.10^6 BMSC/100 μ l of medium. The radiolabelled cells were washed with PBS, which was then added to and mixed with the unlabelled cells.

Implantation procedure The product was injected into the site of NU under radioscopic control using a double trephine (Chirurgical Maintenance, Liège, Belgium), as described by Hauzeur et al. [5]. This device, designed to be used by hand, included a 5-mm external trephine and 3-mm internal trephine, which was 2 cm longer than the external trephine. Under loco-regional or general anaesthesia, a 5-mm skin incision was made. Under fluoroscopic control, the trephine equipped with a guide wire was inserted by hand through the subcutaneous tissue and into the gap between the bone fragments (Fig. 1). After removing the guide wire, the inner trephine was introduced into the fibrotic tissue in order to create a cavity in the NU space for implanting the product (Fig. 1). The product was implanted at a mean dose of 31.7×10^6 cells (range 4–87) in a mean volume of 2.2 ml (range 1.9–3.1 ml). To avoid any leakage, a piece of Gelfoam® absorbable gelatin sponge (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) was pushed through the trephine so as to close the hole. In this study, only one injection was performed in each case, even when larger NU areas were involved.

Fig. 1 Fluoroscopic view of the trephine inserted through the subcutaneous tissue into the gap between the bone fragments of a distal femoral metaphyseal NU fracture

Methods of evaluation The anterior and posterior whole-body planar images (matrix $256 \times 1,024$, speed 3 cm/min) were obtained four, 24 and 48 hours post-injection.

To assess for leakage, a visual analysis of the image after four hours was performed, using a four-point scale: score 0 = none outside the NU site; 1 = some leakage just around the NU site; 2 = more outside than inside the NU site, but not outside the treated bone; and 3 = more outside than inside the NU site and outside the treated bone.

To assess the diffusion and homing process of the labelled cells, regions of interest (ROI) were drawn on the trunk and injection site. The ROI drawn on the injection site included all pixels at 10 % of the maximum activity. On the trunk, the ROI was delineated visually to include the lungs, liver and spleen. For all ROIs, the geometric mean of the counts was recorded. Activity ratios (injection site/body) were calculated at four, 24 and 48 hours. For the injection site, the number of counts at 24 and 48 hours was corrected for decay and then compared against the four hour count.

Relationship with bone reconstruction The progress to bone healing was evaluated by the same observer, an expert in bone radiology, for all patients using a four-point scale: score 0 = no bone reconstruction; 1 = some bone islands; 2 = some bone bridges; and score 3 = complete bone fusion. The time taken to obtain scores 2 and 3 was compared with the leakage score along with the mean radioactive activities at the NU site.

Results

Evaluation of radioactivity retention and the effects of ¹¹¹indium labelling on cellular viability

The mean labelling efficiency for ¹¹¹indium-oxine-labelled osteoblasts ($n=9$) was 78.8 ± 4.6 %. At three and 18 hours, 89.4 ± 21 % ($n=9$) and 67.3 ± 4.7 % ($n=5$) of the initial radioactivity present in the cells was retained intracellularly, respectively. Cell viability

assessed three hours after labelling was 93.7 ± 0.6 % ($n=8$). Although no measurements were undertaken, the day-by-day observation using an inverted microscope suggested a possible decrease in the proliferation rate of labelled cells, whereas the morphology of cells was deemed similar for labelled and unlabelled control cells, as was the alkaline phosphatase staining visualised at day seven of culture.

Evaluation of leakage

Acquisition was achievable in all cases. Visual analysis showed the absence of significant leakage from the injection site in 16 cases (Fig. 2a). In four cases, only grade 1 leakage was observed (Fig. 2b).

Evaluation of osteoblast homing in patients

During the study, several technical problems arose. In two cases, there were no correctly acquired images. In another two cases, some of the acquisitions were lacking: one at four and 48 hours and the other at 24 and 48 hours. All patients showed uptake at the injection site, with four having no other uptake. Four patients showed additional uptake in the bladder, liver and spleen, while 11 patients had additional uptake in the lungs as well as the bladder, liver and spleen (Fig. 3).

The activity ratios (injection site/body) were 48 ± 28 % at four hours, 40 ± 25 % at 24 hours and 35 ± 25 % at 48 hours (Fig. 4a).

Fig. 2 **a** Grade 0 leakage in a right clavicle NU fracture. **b** Grade 1 leakage in a femoral neck NU fracture, suggesting intramedullary leakage

After correcting for decay, the activity within the injection site was 82 ± 15 % at 24 hours and 69 ± 11 % at 48 hours compared with the activity measured at four hours (Fig. 4b).

Relationship between leakage and final NU healing

A radiological NU healing score of 2 was obtained in 16 of the 20 cases in a mean time of 2.5 months (range one to four months) and a score of 3 was obtained in 15 of the 20 cases in a mean time of 5.8 months (range 1–12 months). The five failures did not present any leakage. In contrast, four of the five cases with a leakage score of 1 exhibited good bone repair. Comparing the leakage group with the non-leakage group, the mean time to obtain a radiological score of 2 was 2.5 versus two months and for a score of 3 it was four versus 7.4 months, respectively.

Mean activity (%) at the site in the good bone repair group compared with the failure group was 44 ($n=13$, range 3–85) versus 60 ($n=4$, range 23–82) at 4 h, 65 ($n=13$, range 29–85) versus 73 ($n=3$, range 66–80) at 24 h and 32 ($n=11$, range 5–75) versus 49 ($n=2$, range 26–73) at 48 hours, respectively.

Discussion

The traceability of implanted cells is a challenge for cell-based therapy. The use of $^{111}\text{indium-oxine}$ was previously

Fig. 3 In the case of a left clavicle NU fracture, uptake at 4 h was present in the injection site as well as in the lungs, bladder, liver and spleen

found to be an efficient method for the *in vitro* labelling of cells [6]. *In vivo*, this method was better than technetium-99m and fluorine-18 for the radiolabelling of T lymphocytes in biodistribution studies [7].

This study tested osteoblast cells. *In vitro*, we did not find any adverse effects in terms of viability, morphology or activity except a possible decrease in the proliferation rate, but this observation was not quantified. This cellular toxicity may be accounted for by Auger electrons emitted by indium within very short distances equivalent to the cell diameter [8]. Bindslev et al. found that $^{111}\text{indium}$ -tropolone labelling did not modify the viability of human mesenchymal stem cells (MSC) *in vitro*, their proliferation and ability to differentiate into endothelial cells [9]. Nowak et al.

reported that $^{111}\text{indium}$ -oxine labelling reduced the viability, proliferation and migration of the murine haematopoietic progenitor [10]. In addition, Gholamrezanezhad et al. found a late effect on MSC, with a negative correlation between radiotracer dosage and viability observed at the two week follow-up and only in one bone marrow donor rather than during the early phases at 24 hours, 48 hours, five days or seven days [11]. However, this point still needs to be confirmed and quantified.

The method used for implantation was developed following previous reports for core biopsy [5] and concentrated bone marrow implantation in ON of the femoral head [1]. Both feasibility and safety were found to be excellent. No complications, serious post-operative pain or other serious side effects were reported, and the post-operative morbidity rate was low. Furthermore, the results presented here showed that cells were implanted into the NU site without much leakage, with only local spreading noted. This spreading was probably due to some cancellous bone connections with the NU area, as no extrasosseous extravasations were found.

Concerning the monitoring of the uptake, our results showed a good homing of the implanted labelled cells in the NU site, with 82 % of local activity at 24 hours and 69 % at 48 hours compared with the activity measured at four hours and after correcting for decay. These results are suggestive of early local adhesion of osteoblasts. During the *in vitro* culture in T-flasks, the adhesion of the osteoblasts was observed within two to four hours. In some cases, an increase in uptake in the lung was also found and more often in the bladder, liver and spleen. The activity ratios between

Fig. 4 **a** Activity ratio of injection site to body at 4, 24 and 48 h. **b** Indium activity reduction at 24 and 48 h compared with the activity measured within the injection site at 4 h after correcting for decay

the injection site and the body ranged from 48 ± 28 % at four hours to 35 ± 25 % at 48 hours. The extra site activity is probably associated, at least partly, with the release of free ^{111}In , as observed in vitro. In vitro, only 67 % of the activity remains in the cells at 18 hours. So, what about the quantifications at later times in vivo? The release of free ^{111}In or $^{111}\text{indium-oxine}$ could be even greater in vivo than in vitro. In some in vivo studies using a murine model, $^{111}\text{indium-oxine}$ uptake was detected in the lungs, spleen, bone marrow, liver and kidneys [10, 12].

Finally, our study found no relationship between intraosseous leakage or local uptake and the final bone repair. A larger study is required to confirm these findings. The number of cases was indeed too small for statistical analysis.

In conclusion, $^{111}\text{indium-oxine}$ labelling of the osteoblast cells appears safe, although the proliferation rate needs to be reassessed. Our method for implanting cells into the NU site appears to be validated, avoiding therapeutic bias by cell leakage. In addition, this study's results suggest that the homing of the implanted osteoblast cells with this method was good.

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Conflict of interest The authors declare that they have no conflict of interest.

References

- Gangji V, Hauzeur JP, Matos C, De Maertelaer V, Toungouz M, Lambermont M (2004) Treatment of osteonecrosis of the femoral head with implantation of autologous bone-marrow cells. A pilot study. *J Bone Joint Surg Am* 86-A:1153–1160
- Hauzeur JP, Gangji V (2010) Phases 1–3 clinical trials using adult stem cells in osteonecrosis and nonunion fractures. *Stem Cells Int* 26:410170. doi:10.4061/2010/410170
- Gangji V, De Maertelaer V, Hauzeur JP (2011) Autologous bone marrow cell implantation in the treatment of non-traumatic osteonecrosis of the femoral head: five year follow-up of a prospective controlled study. *Bone* 49(5):1005–1009. doi:10.1016/j.bone.2011.07.032
- Megas P (2005) Classification of non-union. *Injury* 36(Suppl 4):S30–S37. doi:10.1016/j.injury.2005.10.008
- Hauzeur JP, Orloff S, Taverne-Verbanck J, Pasteels JL (1986) Diagnostic of aseptic osteonecrosis of the femoral head by percutaneous transtrochanterian needle biopsy. *Clin Rheumatol* 5:346–358
- McAfee JG, Thakur ML (1976) Survey of radioactive agents for in vitro labeling of phagocytic leukocytes. II. Particles. *J Nucl Med* 17(6):488–492
- Botti C, Negri DR, Seregini E, Ramakrishna V, Arienti F, Maffioli L, Lombardo C, Bogni A, Pascali C, Crippa F, Massaron S, Remonti F, Nerini-Molteni S, Canevari S, Bombardieri E (1997) Comparison of three different methods for radiolabelling human activated T lymphocytes. *Eur J Nucl Med* 24(5):497–504
- Jonsson AC, Jönsson B, Strand SE, Grafström G, Spanne P (1996) Cell survival after Auger electron emission from stable intracellular indium exposed to monochromatic synchrotron radiation. *Acta Oncol* 35:947–952
- Bindslev L, Haack-Sørensen M, Bisgaard K, Kragh L, Mortensen S, Hesse B, Kjaer A, Kastrup J (2006) Labelling of human mesenchymal stem cells with indium-111 for SPECT imaging: effect on cell proliferation and differentiation. *Eur J Nucl Med Mol Imaging* 33(10):1171–1177. doi:10.1007/s00259-006-0093-7
- Nowak B, Weber C, Schober A, Zeiffer U, Liehn EA, von Hundelshausen P, Reinartz P, Schaefer WM, Buell U (2007) Indium-111 oxine labelling affects the cellular integrity of haematopoietic progenitor cells. *Eur J Nucl Med Mol Imaging* 34(5):715–721. doi:10.1007/s00259-006-0275-3
- Gholamrezanezhad A, Mirpour S, Ardekani JM, Bagheri M, Alimoghadam K, Yarmand S, Malekzadeh R (2009) Cytotoxicity of ^{111}In -oxine on mesenchymal stem cells: a time-dependent adverse effect. *Nucl Med Commun* 30(3):210–216. doi:10.1097/MNM.0b013e328318b328
- Cui J, Wahl RL, Shen T, Fisher SJ, Recker E, Ginsburg D, Long MW (1999) Bone marrow cell trafficking following intravenous administration. *Br J Haematol* 107(4):895–902. doi:10.1046/j.1365-2141.1999.01779.x