Real-time imaging of myoblast transplantation using the human sodium iodide symporter

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The quantification of the graft success is a key element to evaluate the efficiency of cellular therapies for several pathologies such as Duchenne muscular dystrophy. This study describes an approach to evaluate the success of myoblast transplantation (i.e., survival of the transplanted cells and the muscle fibers formed) by real-time imaging. C2C12 myoblasts were first transfected with a plasmid containing the human sodium iodide symporter (hNIS) gene. Specific uptake of the radioactive sodium pertechnetate (Na\(^{99m}\)TcO\(_4\)) by the hNIS-positive myoblasts was demonstrated in vitro, while only background level of Na\(^{99m}\)TcO\(_4\) was observed within the control cells. The cells were then transplanted into the tibialis anterior (TA) muscle of mdx (X-linked dystrophic) mice. Following intraperitoneal administration of Na\(^{99m}\)TcO\(_4\), scintigraphies were performed to detect hNIS-dependent Na\(^{99m}\)TcO\(_4\) uptake within the TA. This approach permitted to evaluate the progression of the transplantation and the graft success without having to biopsy the animals during the follow-up period.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive myopathy characterized by the absence of dystrophin (1), a 427-kDa protein that connects the sarcomeric actin to a glycoprotein complex in the cell membrane of muscle and nerve cells (2). Its role in the skeletal muscle is to preserve the integrity and alignment of the plasma membrane to the myofibrils during muscle contraction and relaxation. Transplantation of myoblasts, myogenic precursor cells, is a potential approach to restore dystrophin within DMD patient muscles (3), and the quantification of the success of this graft (i.e., the survival and proliferation of the transplanted cells and the persistence of the muscle fibers that they have formed) is important to evaluate the efficiency of this potential DMD treatment. Here we describe a scintigraphic approach using the human sodium iodide symporter (hNIS) and the radioactive sodium pertechnetate (Na\(^{99m}\)TcO\(_4\)) to follow the proliferation and death of myoblasts transplanted within the mdx mouse skeletal muscle. The hNIS is a thyrocyte channel that plays a key role in iodination and is thought to be critically involved in several thyroid disorders associated with altered iodine uptake (4). It is now well established that there is no expression of hNIS in the skeletal muscles, whereas it is largely expressed in the thyroid gland (5). Thus, C2C12 myoblasts (6) were transfected with a CMV-hNIS-Ires-Neo vector (graciously provided by Stephen J. Russell, Mayo Clinic and Foundation, Rochester, MN) to make them express the hNIS. The neomycin-resistant myoblasts were then selected and transplanted in the mouse tibialis anterior (TA). Intraperitoneal injections of Na\(^{99m}\)TcO\(_4\) (with a short half-life of 6 h) permitted us to quantify the Na\(^{99m}\)TcO\(_4\) uptake within the transplanted myoblasts using a \(\gamma\)-camera. Recent data demonstrated that in control conditions, using systemic injection of Na\(^{99m}\)TcO\(_4\), a nonsignificant part was taken up within the heart and skeletal muscles (7). This technique can thus be used to assess repetitively in vivo the myoblast graft success at different times after the transplantation without having to sacrifice the animal. This approach will facilitate the evaluation of the efficacy of different modifications to improve this treatment in the mdx mouse and in larger animal models.

MATERIALS AND METHODS

Fetal bovine serum (FBS) was purchased from Biomedia (Drummondville, Québec, Canada), metafectene was obtained from Wisent (St. Bruno, Québec, Canada), penicillin and streptomycin were obtained from Gibco (Burlington, Ontario, Canada), Hank’s balanced salt solution (HBSS), trypsin, and Dulbecco’s modified Eagle medium (DMEM) were purchased from Sigma (St. Louis, MO, USA), mouse anti-human hNIS was obtained from Neomarker (Fremont, CA, USA), and goat anti-mouse immunoglobulin G (IgG) conjugated with Alexa™ 546 was purchased from Molecular Probes (Eugene, OR, USA). The Na\(^{99m}\)TcO\(_4\) was provided by the radiology unit of the Centre Hospitalier de l’Université Laval (CHUL; Québec, Canada).

Experimental Protocol

This work was authorized and supervised by the Laval University Animal Care Committee and the Radio Protection Committee of the CHUL. The manipulations were conducted according to the guidelines set by the Canadian Council of Animal Care and Radio Protection Committee.

In Vitro Assay

The C2C12 cell line was obtained from ATCC (Manassas, VA, USA). These cells were originally subcloned from a mouse myoblast cell line (6) able to form contractile myotubes and produce characteristic muscle proteins (8). The C2C12 cells were transfected with a plasmid containing the hNIS gene in the presence of metactenect (with a ratio of 1 µg DNA/5 µL metactenect). They were then selected in DMEM supplemented with 10% FBS, 500 µg/mL neomycin, and 1% penicillin and streptomycin for 1 week. The expression of hNIS within the C2C12-resistant cells was confirmed by immunocytochemistry using a mouse anti-human hNIS and a goat anti-mouse IgG conjugated with Alexa 546. The cells were also cultured in a low serum media (3% FBS) to induce myotube formation in vitro. The C2C12 hNIS-positive cells were also cultured in a T\(_{35}\) flask in the presence of 300 µCi Na\(^{99m}\)TcO\(_4\) for 3 h to investigate their ability to incorporate the sodium pertechnetate.
**In Vivo Assay**

The C2C12 cells expressing the hNIS gene were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. The day of the transplantation, the cells were detached using 0.125% trypsin and washed three times with HBSS. These cells were kept on ice until transplantation (<1 h). Cell pellets were obtained by centrifugation at 1500 rpm for 5 min. Before the transplantation, cell viability was verified using the trypan blue dye, and the cellular count showed that 99.9% of the C2C12 cells were alive. For transplantation, the skin was opened to expose the TA muscle, and the myoblast pellets, resuspended in 20 µL HBSS, were slowly injected throughout the TA muscles.

**γ-Scintigraphies**

Three hours before the scintigraphy, the mice received an intraperitoneal injection of 300 µCi Na\(^{99m}\)TcO\(_4\). Mice were then anesthetized with a 0.1 mL/10 g mixture of 15 mg/mL ketamine and 1 mg/mL xylazine and then taped face down in the bottom of a Plexiglas® box. They were then rapidly transported to radiology unit where they were exposed to the \(\gamma\)-camera. The \(\gamma\)-camera used was a three-heads IRIX made by Marconi-Phillips (Cleveland, OH, USA). The collimator used is a low-energy high-resolution (LEHR). In order to avoid the mice waking, the procedure was accomplished in <40 min. The camera collimator was placed under the Plexiglas box. The hNIS-positive signals coming from the right leg appeared on the left-hand side of the mouse scintigraphies. The interest zone acquired by the computing system corresponding to the transplanted muscle was quantified by analyzing the pixel intensity present within the same interest zone (enlarged black box) using the Scion Image for Windows v.4.0.2 software.

**Statistical Analysis**

Statistical analyses were performed using an analysis of variance (ANOVA) test with the Stat View 512 software (Brain Power, Calabasas, CA, USA)

**RESULTS AND DISCUSSION**

**Determining the Precision of the \(\gamma\)-Camera and the Best Parameters to Make In Vivo Images**

In order to determine the precision of the \(\gamma\)-camera, two different approaches were used. First, two points were plotted using 50 µCi Na\(^{99m}\)TcO\(_4\) for each one. These points were separated by 2.0, 1.0, 0.5, 0.2, and 0.1 cm on millimetric paper. The paper was then exposed to the \(\gamma\)-camera for 10 min. The second procedure consisted in the acquisition of images of a radioactive source (50 µCi Na\(^{99m}\)TcO\(_4\)) through a plumb striated filter called bar phantom. The plumb bands (white bands) were separated by 2.0, 1.0, 0.5, and 0.2 cm (9). These two procedures permitted us to evaluate the capacity of the optical or electronic equipment to transfer signal faithfully by verifying how well alternating white and black strips or points showed-up (10). These results are shown in Figure 1.

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**Figure 1. Evaluation of the best parameters to be used to make in vivo images.** To determine the exposition time and contrast to be used in vivo, 500,000 human sodium iodide symporter (hNIS)-positive C2C12 cells were transplanted in the right tibialis anterior (TA), whereas the left TA was injected with the same number of hNIS-negative myoblasts. Representative images of mice acquired 24 h after the transplantation using two exposition times and three contrast phases (40%, 10%, and 3%) are shown. The difference between the muscle injected with labeled and unlabeled cells was better detected at 3% contrast and 10-min acquisition. A relative \(\gamma\)-emission scale is provided to make visual appreciation of the intensity of the scintigraphy.
tests demonstrated that the γ-camera maximal resolution was 2 mm and that below this distance two radioactive points were interpreted by the acquisition system as only one (data not shown).

Preliminary experiments were also performed within three mice to determine the best contrast and exposition duration to be used to make in vivo images obtained with the γ-camera (Figure 1). Different exposition times and three contrast phases were tested on the mdx mouse transplanted 1 day earlier with 500,000 C2C12 cells within each TA. The C2C12 cells were previously transfected with a CMV-hNIS-Ires-Neo vector and selected for 20 days with DMEM supplemented with 500 µg/mL neomycin, 10% heat inactivated FBS, and 1% penicillin and streptomycin. Figure 1 shows that at 10 min of exposition and at 3% of phase contrast, there was a significant difference between the right TA (transplanted 1 day before with hNIS-positive C2C12 cells) and the left TA (transplanted with hNIS-negative C2C12 cells). The signal-to-noise ratio of the control leg versus the host leg was relatively modest at 1 day. This result could be explained by the fact that the blood vessels in the injection zones in both the experimental and the control muscles were damaged and the label may be caught nonspecifically in a blood clot 1 day after the transplantation. The difference between these two muscles was less visible after 5 min of exposition. The organs that gave strong signals were the salivary and thyroid glands (head) and the stomach (abdomen region).

### C2C12 Cells Expressing the hNIS Gene Are Able to Form Myotubes In Vitro

This experiment was performed to verify whether the myoblasts expressing the hNIS gene preserved their ability to differentiate in myotubes. The expression of hNIS on C2C12 cells and on differentiated myotubes was evaluated by immunocytochemistry (Figure 2A) following neomycin selection and fusion in a low serum media (DMEM 3% serum). This labeling demonstrated that 95% of the cells were hNIS-positive. These results also indicated that the myoblasts expressing the hNIS were able to form myotubes. Moreover, an in vitro test was performed to determine the ability of hNIS-positive cells to uptake $\text{Na}^{99m}\text{TeO}_4$. The hNIS-positive and -negative C2C12 cells were plated in T_75 flasks at 90% confluence. These cells were then washed with HBSS and incubated with 300 µCi $\text{Na}^{99m}\text{TeO}_4$ for 3 h. The cells were then washed five times with HBSS and analyzed using the γ-camera. Figure 2B demonstrates that the hNIS-positive cells were able to uptake $\text{Na}^{99m}\text{TeO}_4$ in vitro, while only a low background level of absorption was observed within the nontransfected C2C12 cells.

### In Vivo Evaluation of the Proliferation of the Transplanted Myoblasts

A long-term in vivo evaluation of the proliferation of the transplanted myoblasts was also performed. Two quantities of C2C12 cells (500,000...
or 1,000,000) were transplanted in different TA. The right TA were transplanted with hNIS-labeled cells while the left TA received unlabeled cells. The amount of γ-labeling due to the presence of these transplanted cells was then evaluated in the same mice (n = 4) 3 h, 3 days, and 1, 2, 3, and 4 weeks later (Figure 3). Three hours before the scintigraphies, the mice received 300 µCi Na\textsuperscript{99m}TcO\textsubscript{4}. The thyroid (100% emission) and the tail (0% emission) were taken as references to compare the muscle relative intensity. Three hours after the transplantation, an 8.6% difference was detected between the right and the left TA, following the injection of 500,000 C2C12 cells and an 11.6% difference with 1,000,000 cells. In spite of this mean difference, statistical analysis showed that the uptake in the right and left TA was not significantly different. A possible hypothesis to explain this lack of difference is that the myoblasts were not able to effectively uptake the Na\textsuperscript{99m}TcO\textsubscript{4} due to the formation of a blood clot. To investigate this hypothesis, two different experiments were performed. First, 10\textsuperscript{6}, 2 × 10\textsuperscript{6}, 3 × 10\textsuperscript{6}, and 4 × 10\textsuperscript{6} C2C12 cells were injected into mice TA. The result showed that the Na\textsuperscript{99m}TcO\textsubscript{4} uptake was not improved 3 h after the graft, when the transplanted cell number was increased (data not shown). In a second experiment, a dye (diacetyl fluorescein) was injected to the mice transplanted with 1,000,000 C2C12 cells. The animals were killed at day 0 (3 h) and 1, 2, and 3 days after the cell transplantation, and the injected

Figure 3. In vivo evaluation of survival and/or proliferation of transplanted myoblasts. The right tibialis anterior (TA) was injected with human sodium iodide symporter (hNIS)-positive cells, while the left TA was transplanted with nontransfected cells. Three hours before the scintigraphies, mice were injected with 300 µCi of the radioactive sodium pertechnetate (Na\textsuperscript{99m}TcO\textsubscript{4}). Representative radiographs of transplanted mice obtained at 40%, 10%, and 3% contrast phases are illustrated. The panels represent the C2C12 cell state 3 h, 3 days, 1 week, 2 weeks, 3 weeks, and 4 weeks after the transplantation. Within each scintigraphy, the upper photos show mice transplanted with 500,000 C2C12 cells, and the lower images represents mice injected with 1,000,000 C2C12 cells. A relative γ-emission scale is provided to make visual appreciation of the intensity of the scintigraphy.
TA muscles were frozen, sectioned, and analyzed. The microscopy analysis demonstrated that the diacetyl fluorescein was absorbed by the cells at each analysis time (data not shown). Thus, the increased labeling difference between 3 h and 3 days is not due to the vascularization of transplanted cells. An alternative hypothesis is that the hNIS was damaged by the trypsinization, and the cells were not able to take up the radioactive label until the new hNIS proteins were expressed, which took a few days to occur. Three days after the transplantation, the differences between the control and the test muscles were significantly increased: the muscles transplanted respectively with 500,000 and 1,000,000 cells were 15.2% and 23.2% (Table 1) more labeled than their control TA. The increased labeling differences between 3 h and 3 days could perhaps be explained by the proliferation of transplanted cells. This hypothesis does not seem sufficient, since no difference was detected between 10⁶ and 4 × 10⁶ cells at 3 h. The results support the alternative hypothesis that the hNIS was damaged, and that shortly after the transplantation, the cells were unable to take up the marker. The differences between the control and the test muscles were further increased during the 4 weeks following the transplantation. Indeed, 1 week after the graft, the differences between the right and the left TA using 500,000 and 1,000,000 cells were, respectively, 22.0% and 35.0%, at 2 weeks the differences were 36.1% and 45.6%, and at 3 weeks the differences were 54.2% and 48.8%. At 4 weeks, the differences were 56.1% and 58.3% (Table 1). This increasing difference between the muscles injected with hNIS-transfected cells and the control muscle is probably due to cell proliferation. However, there is probably a limited proliferation of these cells that with time attenuates the differences between the muscles injected with 500,000 or 1,000,000 cells (Table 1).

These results indicate that cell labeling with an hNIS gene permits to follow the cells transplanted within organs and tissues, which do not normally express the sodium iodide symporter. This method could eventually permit to evaluate in vivo the cell survival and proliferation. This technique can be used not only in skeletal muscles but also within the heart, where there is also no expression of hNIS. In fact, some techniques have been tested to evaluate the efficiency of anti-apoptotic (11,12) and immunosuppressive (13,14) treatments for myoblast transplantation. Using this hNIS-labeling technique, there would be no need to kill the animals to estimate the myoblast survival or the effectiveness of immunotolerance induction on the transplantation success. The size precision of the method can be improved by using a magnetic resonance imaging (MRI) instrument specifically developed for mice rather that a clinical instrument as used in our experiment. Moreover, when used on larger animal models, such as the dystrophic dogs that represent one of the best DMD animal models (15,16), this procedure could be used to evaluate graft success by making three-dimensional images of transplanted muscles. Finally, this labeling method can be used to study the survival and proliferation of other types of cells such as islets and hepatocytes.

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### COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

### REFERENCES


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