Non-viral gene transfer to the tendon: comparison of two methods

Transferência gênica não viral para tendão: comparação de dois métodos

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Abstract

Background: tendons are part of the connective tissue that joins muscle to bone. Tendon injuries are a problem, since they have a poor ability to regenerate spontaneously. Alternative treatments involving the injection of local growth factors and gene transfer has been evaluated. Thus, we compared two methods for non-viral gene transfer tendons, using the GFP gene as reporter gene.

Methods: Wistar rats had the medial quadriceps tendon exposed and the plasmid was transferred by direct injection or complexed with liposomes. Quantification of GFP in the tendom and in the spleen was evaluated by histological analysis with a fluorescence microscope.

Results: gene transfer to the tendon was successfully obtained in both treatments. Lipoplex, as expected, showed the highest efficiency in transducing tenocytes, however we have found GFP expression also in the spleen. Naked DNA also showed fluorescence values above the control group and the signal was limited to the tendom.

Discussion: the use of GFP as a reporter gene is a classical approach to evaluate gene transfer efficiency. Non-viral gene transfer methods are safe but show low levels of transduction and transient expression. For tendon repair, however, these characteristics may prove beneficial because a transient expression may be desirable to avoid the risk of adverse effects. GFP distribution in the spleen was probably a result of lipoplexes uptake by cells from the reticular endothelial system.

Conclusion: taking into account the distribution of GFP in another tissue when using lipoplex, we believe that naked DNA is a more appropriate way to perform gene transfer to the tendon, ensuring safety, low cost and easy handling.

Keywords: tendon injury; GPF expression; gene transfer

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Resumo

Introdução: injúrias no tendão representam um problema, uma vez que estes têm pobre capacidade de regeneração espontânea. Tratamentos envolvendo injeção local de fatores de crescimento e transferência gênica tem sido avaliados. Assim, comparamos dois métodos de transferência gênica não viral para tendões, usando o gene GFP como gene repórter.

Métodos: ratos Wistar tiveram a porção medial do tendão quadriciptal exposto e o plasmídeo foi transferido através de injeção direta ou complexado com lipossoma. A quantificação de GFP no tendão e no baço foi avaliada por análise histológica.

Resultados: a transferência gênica para o tendão foi obtida com sucesso nos dois tratamentos. Lipoplexo demonstrou maior eficiência na transfecção, porém a presença de GFP foi detectada também no baço. A transfecção com DNA nu demonstrou valores de fluorescência superiores ao grupo controle e o sinal foi limitado ao tendão.

Discussão: o uso de GFP como gene repórter é uma abordagem clássica para avaliar a eficiência da transferência de genes. A transferência não-viral é segura embora apresente expressão transiente. Para o reparo do tendão, no entanto, essas características podem ser benéficas, pois uma expressão transiente pode ser desejável para evitar o risco de efeitos adversos. A distribuição de GFP no baço foi provavelmente resultado da absorção dos lipoplexos por células do sistema retículo endotelial.

Conclusão: twendo em conta a distribuição de GFP em outro tecido quando utilizamos lipoplexo, pensamos que o DNA nu é uma forma mais adequada para realizar a transferência de genes para o tendão, garantindo segurança, baixo custo e fácil manuseio.

Palavras-chave: lesões no tendão; expressão de GFP; transferência gênica

Tendons are part of the connective tissue that joins muscle to bone (1). They are dense bands that help in maintaining the normal mobility of the joints. Less than 5% of its total volume is constituted by specialized cells, the tenocytes.

Tendon disorders are frequent in sport and occupational medicine. Achilles tendinopathy is the most common running-associated tendinopathy followed by runner's knee and shin splints (2). Tendon lesions also affect 5-10% of people with more than 65 years old (3). These injuries are a serious clinical problem, as tendons have very poor spontaneous regenerative capabilities. Complete regeneration is never achieved and the strength of tendon and ligaments remain as much as 30% lower than normal even months or years following an acute injury (4).

However, tendons seem to be the least complex of the connective tissues with respect to their composition and architecture and this leads to the expectation that they would be more amenable to tissue engineering approaches than other tissues (4). These treatments involve local injection of stem cells (5) and gene therapy (6). Gene transfer could improve the repair process, by permitting local production of therapeutic substances, e.g. growth factors. However, the major concern remains transferring therapeutic genes in effective, safe and target-directed manner (7,8).

In this study, two in situ non-viral methods of gene transfer (naked DNA and Lipoplex approaches) to the quadricipital tendon were compared, using the green fluorescent protein (GFP) as reporter gene.

Methods

Animals

Ten male Wistar rats (Rattus norvegicus) weighing 175-200 g were anesthetized with 0.2 mL of xylazine chlorydrate (2.3%) and 0.1 mL of ketamine chloridrate (11.6%). To perform the gene transfer the left quadricipital tendon was exposed and cut transversely at the central portion. After the gene transfer, the fascial tissue and the skin were sutured and animals were allowed to return to cages. Animals were sacrificed in CO2 chamber four days after surgery. The quadricipital tendon and the spleen were dissected and prepared for histological analysis.

During the experiment, animals were housed at the Animal Experimental Unit (Experimental Research Center-HCPA) under controlled temperature (between 18 and 22ºC) in light-dark cycles of 12 hours. Standard rat chow and water were given ad libitum. All animal procedures followed international guidelines for the care and use of laboratory animals and this project was approved by the research ethics committee (GPPG 03-025).

Gene transfer

Two non-viral methods of transfection were compared: lipofection using Lipofectamine2000TM (Invitrogen, USA) and naked DNA. The pTRACER-CMV2 plasmid (Invitrogen-USA), containing the GFP/Zeocin fusion gene under control of CMV promoter was used.

Lipoplex preparation used 5 µg of pTRACER-CMV2 combined with Lipofectamine2000TM (Invitrogen, USA), according to manufacturer's instructions. Briefly, 5µg of the pTRACER-CMV2 diluted in 15 µl of TE were added to 10 µl of PLUS reagent and incubated for 15 minutes at room temperature. After that, 20 µl of Lipofectamine2000TM was added and incubated for more 15 minutes under the same conditions. At this moment, the final solution was ready to use. Naked DNA gene transfer was performed by injecting 5 µg of pTRACER-CMV2 plasmid diluted in 15 µl of TE solution.

Animals were divided into 3 groups: the first (n=4) received naked DNA (pTRACER-CMV2 only), the second (n=4) received Lipoplex (pTRACER-CMV2 plus Lipofectamine2000TM), and the third received only 15 µl of TE solution, as negative control (n= 2). Naked DNA, Lipoplexes, and TE only were aspersed directly over the left leg tendon, using an insulin-like syringe, and the efficiency of gene transfer using the two non-viral methods was evaluated by the presence GFP positive cells observed under histological analysis four days later.

Detection and quantification of gene expression

To measure GFP gene expression, the quadricipital tendon and the spleen were dissected and immediately fixed by immersion in PBS solution containing 4% formaldehyde, 7% picric acid and 10% sucrose, for 15 minutes. After fixation, organs were included in Tissue-Tek (OCT) and frozen under liquid nitrogen. Frozen tissue blocks were warmed to -20°C and 7 µm sections were cut on a cryostat. Slides were dried at room temperature and observed under fluorescence microscopy (Olympus BX41®) using the U-MWB2 filter set (BP460-490, DM500, BA520IF).

Photographs of tendon and spleen were analyzed at low power fields (10X magnification). Twelve randomly selected fluorescent fields per slide were photographed with ISO400 films, with a 32 seconds diaphragm aperture time. Then, the images were digitalized and transformed into RGB format. Autofluorescence was eliminated using Corel PhotoPaint 11® software, by selecting the green color as the only one in the visible spectra, thus eliminating the interference of other colors. The amount of green pixels was quantified by subtracting the non-green pixels to the total pixels of the images.

Statistical analysis

The intensity of fluorescence measured by the amount of green pixels was compared by the Kruskal-Wallis test for non-parametric values (level of significance of 0.05), using the Statistical Package for Social Sciences v11.0 (SPSS).

Results

In situ gene transfer to the tendon was successfully obtained with both treatments (Lipoplex and naked DNA), as demonstrated by intense fluorescent cells observed under fluorescent microscopy (figure 1).

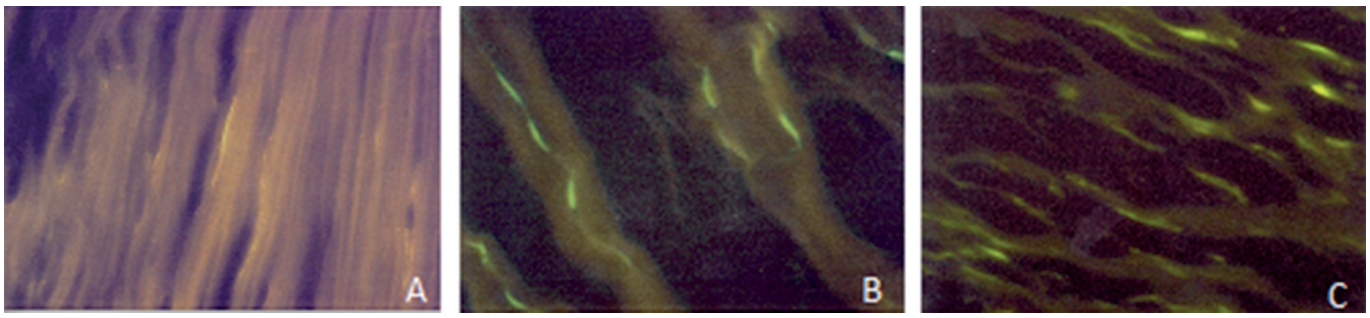

Figure 1: Fluorescent microscopy of the quadricipital tendon (40X). A) Control, B) naked DNA, C) Liploplex.

Surprisingly, GFP positive cells were also observed in the spleen, especially in the Lipoplex group (figure 2).

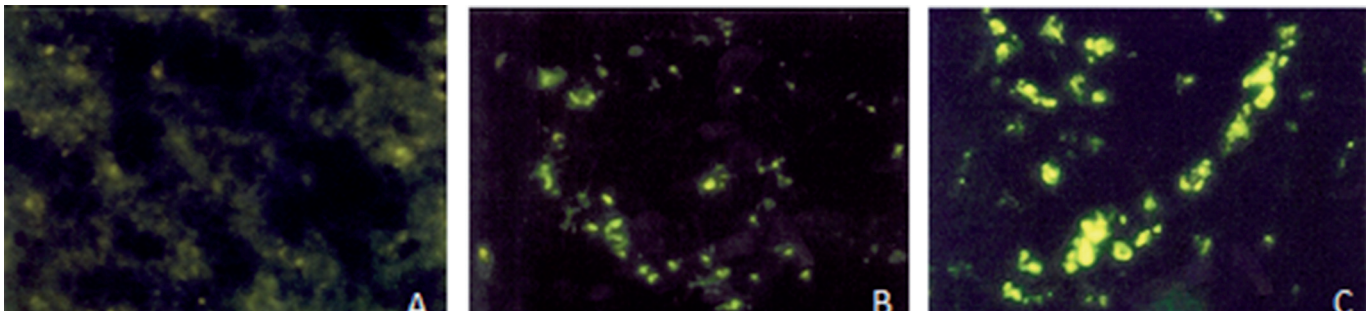

Figure 2: Fluorescent microscopy of the spleen (40X). A) Control, B) naked DNA, C) Liploplex.

Endogenous fluorescence was observed both in tendon and spleen. In tendon images, a visual distinction could be made between GFP and endogenous fluorescence (figure 1A). However, on spleen slides, this distinction was not so clear, probably due to less intense GFP expression (figure 2). The analysis of the images using the computer software eliminates the interference of the endogenous fluorescence, resulting in a gray background, thus emphasizing the green cells (figure 3). This process was performed for tendon and spleen before pixel quantitation.

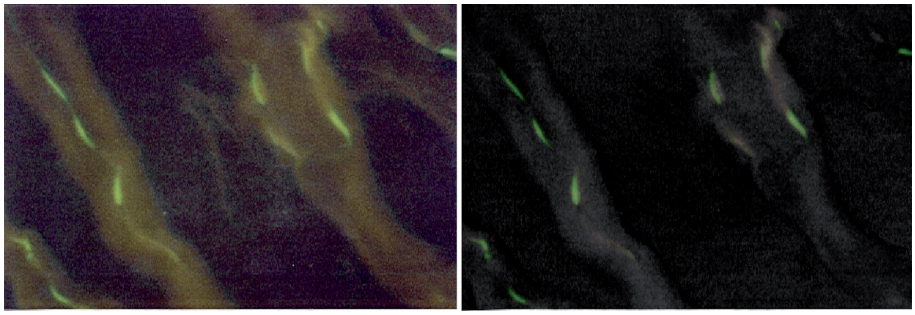

Figure 3: Elimination of autofluorescent background using computer software. A) Fluorescent microscopy of the quadriciptal tendon transfected with naked DNA. B) Same image digitally treated to eliminate the interference of the endogenous fluorescence, resulting in a gray background and emphasizing the green cells.

Data from the digital image analysis is summarized in Table 1. Effective gene transfer to the tendon was achieved using both non-viral vectors. Lipoplex, as expected, showed the highest efficiency in transducing tenocytes,as shown by higher fluorescent levels compared to control and naked DNA ($p<0.001$). However, in the spleen, Lipoplex also showed fluorescence values significantly higher than the other two groups ($p=0.008$ for control group and $p=0.024$ for naked DNA). On the other hand, naked DNA showed fluorescence values above the control group in the tendon ($p<0.001$), but not in the spleen ($p=0.828$).

Table 1: Fluorescence level measured by the number of green pixels in images of tendon and spleen of animals treated either with Lipoplex, naked DNA or TE only (control).

		Lipoplex	Naked DNA	Control
Tendon	Median	0.2749*	0.1387*	0.0158*
	Percentile 25	0.1845	0.0468	0.0022
	Percentile 75	0.3940	0.2436	0.0494
Spleen	Median	0.1677*	0.062	0.0656
	Percentile 25	0.0872	0.0294	0.0280
	Percentile 75	0.2760	0.1562	0.1333

Note: ** denotes statistical difference from other groups ($p< 0.05$, Kruskal-Wallis test for non-parametric values). For specific p values, see text.*

Discussion

In this work we showed the feasibility of gene transfer to tendons using two non-viral gene transfer methods. The use of GFP as a reporter gene is a classical approach to evaluate gene transfer efficiency (9-11). It can be assumed that fluorescence intensity is a marker of gene expression and an indirect measure of gene transfer efficiency. The main difficulty in this case is the presence of endogenous fluorescence, called autofluorescence. For solving this problem, the use of computer programs, as performed in the present work, is common sense (12).

Non-viral gene transfer methods are safe but show low levels of transduction and transient expression. For tendon repair, however, these characteristics may prove beneficial. Most protocols suggest the introduction of growth factors as therapeutic genes (13,14). Thus, a transient and local gene expression may indeed be desirable to prevent the risk of adverse effects such as extracellular matrix deposition or vascular overgrowth (15,16).

In this aspect, the biodistribution of non viral methods is another relevant aspect although not extensively studied. It is generally accepted that DNA degradation in the blood vessels limits the plasmid diffusion and that non viral gene transfer methods are suitable only for in situ delivery (17). Nevertheless, using Lipoplex we have found GFP expression at the spleen. This distribution was probably a result of plasmid/liposome complex uptake by cells from the reticular endothelial system, a phenomenon already described by Burke et al (18). Despite the low expression level observed in the spleen, this unintended targeting of the vector could be solved by the use of tissue-specific promoters controlling the transgene expression just at the desired tissue (19,20). This strategy using tissue-specific promoters has been successfully used in gene transfer for macrophages (21) and in the treatment of tumors (22,23) for example.

Taking into account the distribution of the reporter gene found when liposomes were used; we think that naked DNA is a more suitable way to perform gene transfer to the tendon. In addition, with a protocol using naked DNA, we were able to achieve efficient gene transfer with low costs, easy and safe manipulation.

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Referências

1. Aslan H, Kimelman-Bleich N, Pelled G, Gazit D. Molecular targets for tendon neoformation. J Clin Invest. 2008;118(2):439-44.
2. Knobloch K, Yoon U, Vogt PM. Acute and overuse injuries correlated to hours of training in master running athletes. Foot Ankle Int. 2008;(7):671-6.
3. AAOS. On line service fact sheet the knee. Available from: URL: http://www.aaos.org/
4. Rotini R, Fini M, Giavaresi G, Marinelli A, Guerra E, Antonioli D, et al. New perspectives in rotator cuff tendon regeneration: review of tissue engineered therapies. Chir Organi Mov. 2008;91(2):87-92.
5. Smith RK. Mesenchymal stem cell therapy for equine tendinopathy. Disabil Rehabil. 2008;30(20-22):1752-8.
6. Sharma P, Maffulli N. Tendinopathy and tendon injury: the future. Disabil Rehabil. 2008;30(20-22):1733-45.
7. Giannoudis PV, Tzioupis CC, Tsiridis E. Gene therapy in orthopaedics. Injury. 2006;37 Suppl 1:S30-40.
8. Gouze E, Gouze JN, Palmer GD, Pilapil C, Evans CH, Ghivizzani SC. Transgene persistence and cell turnover in the diarthrodial joint: implications for gene therapy of chronic joint diseases. Mol Ther. 2007;15(6):1114-20.
9. Welsh S, Kay SA. Reporter gene expression for monitoring gene transfer. Curr Opin Biotechnol. 1997;8(5):617-22.
10. Horn C, Schmid BG, Pogoda FS, Wimmer EA. Fluorescent transformation markers for insect transgenesis. Insect Biochem Mol Biol. 2002;32(10):1221-35
11. Oliveira LA, Kim C, Sousa LB, Schwab IR, Rosenblatt M. I Gene transfer to primary corneal epithelial cells with an integrating lentiviral vector. Arq Bras Oftalmol. 2010; 73(5):447-53.
12. Cutroneo KR. Gene therapy for tissue regeneration. J Cellular Bochemistry. 2003; 88:418-25.
13. Lyras DN, Kazakos K, Verettas D, Chronopoulos E, Folaranmi S, Agrogiannis G. Effect of combined administration of transforming growth factor-b1 and insulin-like growth factor I on the mechanical properties of a patellar tendon defect model in rabbits. Acta Orthop Belg. 2010;76(3):380-6.
14. Berglund ME, Hart DA, Reno C, Wiig M. Growth factor and protease expression during different phases of healing after rabbit deep flexor tendon repair. J Orthop Res. 2011;29(6):886-92.
15. Anitua E, Sánchez M, Zalduendo MM, de la Fuente M, Prado R, Orive G, et al. Fibroblastic response to treatment with different preparations rich in growth factors. Cell Prolif. 2009;42(2):162-70.
16. Cawston TE, Young DA. Proteinases involved in matrix turnover during cartilage and bone breakdown. Cell Tissue Res. 2010;339(1):221-35
17. Friedmann T, Rossi J. Gene transfer: delivery and expression of DNA and RNA: a laboratory manual. 1st ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press;2007.
18. Burke B, Sumner S, Maitland N, Lewis CE. Macrophages in gene therapy: cellular delivery vehicles and in vivo targets. J Leukoc Biol. 2002;72(3):417-28.
19. Räty JK, Lesch HP, Wirth T, Ylä-Herttual. Improving safety of gene therapy. Curr Drug Saf. 2008; 3(1):46-53.
20. Kuzmin D, Gogvadze E, Kholodenko R, Grzela DP, Mityaev M, Vinogradova T, et al. Novel strong tissue specific promoter for gene expression in human germ cells. BMC Biotechnol. 2010; 17;10:58.
21. Levin MC, Lidberg U, Jirholt P, Adiels M, Wramstedt A, Gustafsson K, et AL. Evaluation of macrophage-specific promoters using lentiviral delivery in mice. Gene Ther. 2011 [in press].
22. Saukkonen K, Hemminki A. Tissue-specific promoters for cancer gene therapy. Expert Opin Biol Ther. 2004; 4(5):683-96.
23. Niess H, Bao Q, Conrad C, Zischek C, Notohamiprodjo M, Schwab F, et al. Selective targeting of genetically engineered mesenchymal stem cells to tumor stroma microenvironments using tissue-specific suicide gene expression suppresses growth of hepatocellular carcinoma. Ann Surg. 2011;254(5):767-74.
24. Pacak CA, Sakai Y, Thattaliyath BD, Mah CS, Byrne BJ. Tissue specific promoters improve specificity of AAV9 mediated transgene expression following intra-vascular gene delivery in neonatal mice. Genet Vaccines Ther. 2008; 23;6:13.

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