MOLECULAR & CELLULAR TOXICOLOGY, Vol. 3, No. 2, 85-89, June 2007

Efficient Gene Delivery through the Human Transferrin Receptor of Fibroblast-like Synoviocytes Stimulated with bFGF: a Potential Target Receptor for Gene Transduction in Rheumatoid Arthritis

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Accepted 29 April 2007

Abstract

Efficient gene delivery to specific tissues, such as inflammatory and cancerous tissues, is currently a major concern in disease treatment. The human transferrin receptor (hTR) has been detected in the synovium and fibroblast-like synoviocytes (FLS), which raises the possibility that expression of hTR is associated with the pathogenesis of rheumatoid arthritis (RA). To investigate whether the hTR is a useful target for gene transduction into the FLS of RA patients, recombinant adenoviruses with wild-type fiber (AdLac) and transferrin peptide-tagged fiber (Tf-AdLac) were used. The hTR expression level in FLS was notably increased by basic fibroblast growth factor (bFGF). Gene transduction to FLS was significantly higher by the hTR-targeted adenovirus than by the AdLac adenovirus, and treatment of the FLS with bFGF resulted in increased gene transduction by Tf-AdLac. Taken together, these data support Tf-AdLac as a new strategy for gene transduction in the treatment of RA patients.

Keywords: Fibroblast-like synoviocyte, Rheumatoid arthritis, Basic fibroblast growth factor, Human transferrin receptor, Recombinant adenovirus

The human transferrin receptor (hTR) is a type II cell surface receptor that binds to transferrin (Tf), with subsequent internalization of the complex through clathrin-coated pits. The hTR is expressed ubiquitously and is overexpressed at least 100-fold in cancerous tissues of the mouth, liver, pancreas, and prostate, as well as in other tissues[1]. This increase in hTR expression in cancers is attributed to the increased metabolism of transformed cells. Thus, endocytosis of bound Tf to hTR presents a useful target for gene therapy[1]. Rheumatoid arthritis (RA) is a chronic inflammatory disorder that affects the joints and leads to bone destruction[2]. The synovium in RA patients is characterized by the infiltration of various inflammatory cells and excessive growth and migration of synovial cells, which result in joint destruction[3]. Similar to many cancerous tissues, hTR mRNA is expressed by synovial fibroblasts, while the receptor is not expressed in synovial macrophages or synovial fluid neutrophils[4]. The expression of hTR is associated with cell proliferation[1], and the fibroblasts in the rheumatoid synovium are linked to proliferation[5]. In the present study, we hypothesize that hTR can be used as a therapeutic target for gene delivery tools. To enhance the expression of hTR, we used basic fibroblast growth factor (bFGF). We constructed adenoviral vectors that carry transferrin. Among the several viral vectors used for gene therapy, adenovirus serotype 5 (Ad5) vectors have been studied extensively in various models. The Ad5 cell surface receptors are essential for virus infection, and binding of the knob domain of the Ad5 fiber to the high-affinity coxsackie-Ad receptor (CAR) mediates initial attachment[6]. Therefore, a type 5 adenovirus with a modified CAR-binding domain (knob) has been developed[6]. Other modified viruses have been used to retarget neuroglial cells and express the hTR[7]. However, CAR is absent from the human RA synovium[8]. This lack of CAR may account for the low permissiveness to Ad5[8-10]. Therefore, we used bFGF to enhance the expression of hTR, so as to increase the binding efficiency of hTR-targeted adenoviruses (Tf-AdLac) to cultured FLS.

In the present study, we examined the effects of bFGF on hTR expression and investigated whether

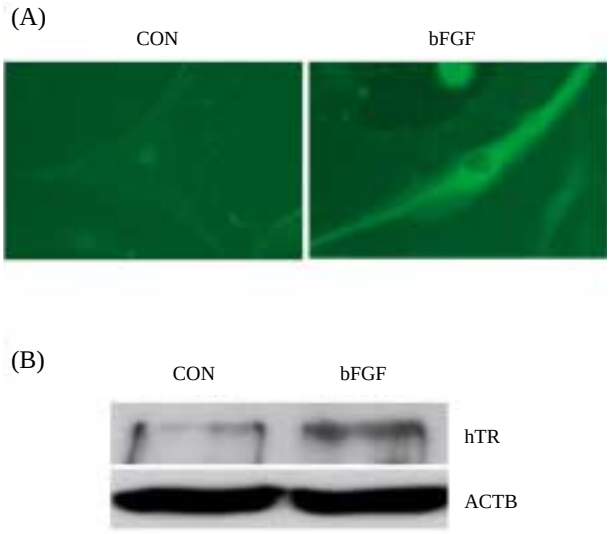

Figure 1. The addition of bFGF increased hTR expression by FLS. FLS were treated with 100 ng/mL bFGF in serum-free DMEM for 24 hr. (A) The expression of hTR was assessed by immunocytochemistry. As assessed by staining with the anti-hTR antibody (1 : 100 dilution), hTR was expressed weakly under normal conditions, whereas after the addition of bFGF, hTR expression was increased (original magnification ×400). (B) Expression of hTR protein determined by Western blotting with the anti-hTR antibody (1 : 100). ACTB was used as an internal control. CON, control; bFGF, 100 ng/mL bFGF; hTR, human transferrin receptor; ACTB, β-actin.

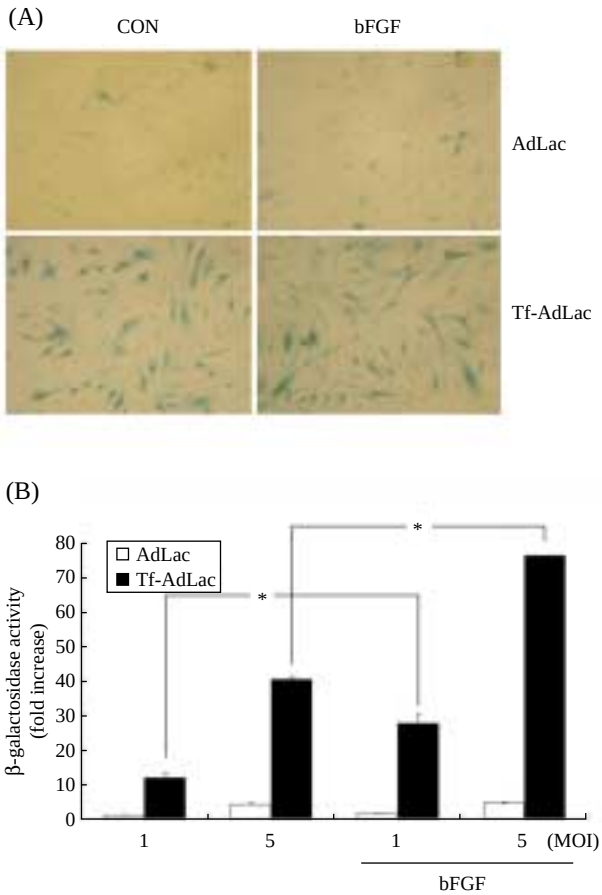

Figure 2. Recombinant adenovirus-mediated gene transduction was enhanced by treatment with bFGF. (A) X-Gal staining shows that the Tf-AdLac virus was more efficient than the AdLac virus in transducing the β-galactosidase gene into cultured FLS, and that this effect was enhanced by the addition of bFGF. Viral infection in this experiment was at an MOI of 5 (original magnification ×200). (B) The addition of bFGF enhanced the infectious efficacies of AdLac and Tf-AdLac. Recombinant adenovirus-mediated gene transduction into FLS was measured by the β-galactosidase activity assay 24 hr after infection with AdLac or Tf-AdLac. The enzymatic activities were higher in cells infected with Tf-AdLac than in those infected with AdLac. These effects were enhanced by treatment of the FLS with bFGF ($P < 0.005$). The data shown represent the average and standard deviation of four independent experiments. The viral MOIs used in these experiments are indicated.

hTR can be used as a useful target for gene delivery, by determining the infectious efficiencies of the Tf-AdLac and wild-type AdLac adenoviruses for FLS.

bFGF Increases the hTR Expression of FLS

The expression of hTR was determined by immunocytochemistry and Western blotting analysis. Immunostaining for hTR showed a remarkable increase in expression in bFGF-treated FLS (Figure 1A, left) compared to the negative control (Figure 1A, right). Western blot analysis confirmed the increased level of hTR in FLS treated with bFGF (100 ng/mL for 24 hr; Figure 1B). These data indicate that bFGF is a key factor in hTR expression by FLS.

Infection of the hTR-targeted Adenovirus into FLS

We examined whether hTR is an efficient target for gene delivery. For targeting hTR, we used an adenovirus that has a transferrin peptide-tagged fiber (Tf-AdLac)[11]. FLS were treated with bFGF (100 ng/mL for 24 hr) or 1% BSA solution, followed by infection with AdLac or Tf-AdLac. After 24 hr of infection with AdLac or Tf-AdLac, the β-galactosidase activities were determined. At a multiplicity of infection (MOI) of 5, gene transduction of FLS was more efficient with Tf-AdLac than with AdLac, as detected by X-Gal staining (Figure 2A). Since most of the FLS infected at MOIs of 10 and 50 exhibited a blue color, these cells could not be assessed for infection efficiency. Therefore, we performed the β-galactosidase assay on cells infected at an MOI of 5. Figure 2B

shows the β-galactosidase activity of the virus-infected FLS, as determined by X-Gal staining assay. The enzymatic activity was higher in cells infected with Tf-AdLac than with AdLac virus alone, and this effect was enhanced by treatment with bFGF.

Discussion

In the present study, we demonstrated that hTR expression in FLS is increased by the addition of bFGF, and that hTR-expressing FLS are more efficiently infected with adenoviruses than non-treated FLS. These results suggest that hTR-expressing FLS may represent an effective target for gene therapy in RA patients.

Although there are several biological agents for the treatment of RA, such as etanercept, infliximab, adalimumab, anakinra, abatacept, and rituximab, their use may be limited by high cost and various side-effects[12-15]. Gene therapy has been developed as an alternative approach to the targeted delivery of therapeutic agents. The Ad5 vectors have been used extensively in animal models of arthritis due to their ability to infect both proliferating and non-proliferating cells and their relative safety[6,8-10,16]. However, human rheumatoid synoviocytes are reported to be poorly permissive to Ad5 vectors due to the absence of CAR[8]. Although the low levels of transgene expression obtained with Ad5 can be compensated by the use of high MOI, this may induce toxicity. Furthermore, the presence of high levels of neutralizing anti-Ad5 antibodies in synovial fluid may limit the use of such vectors in human disease. Thus, strategies to improve Ad5 viral tropism are desirable. Since the expression of hTR in rheumatoid synoviocytes is reported to be relatively weak[2], receptor-targeted viral vectors are less useful. Therefore, increasing the level of hTR on FLS could be an efficient target for hTR-targeted viral vectors in RA patients. To increase hTR expression, we have screened some candidate molecules and chemicals that are known as inducers of hTR. Of these molecules, only bFGF induced an increase in hTR expression on FLS (data not shown). bFGF is one of the important peptides in the pathogenesis of RA. Furthermore, bFGF is one of the heparin-binding growth factors that induce potent mitogenic effects on various cells of mesodermal and ectodermal origin. Higher levels of bFGF have been detected in the synovial fluid of RA patients compared to OA patients[17,18]. Based on these studies, we treated the FLS with bFGF and found that the level of hTR expression was increased significantly, as assessed by immunocytochemistry and Western blotting (Figure 1). We then investigated whether these hTR-overexpressing cells were suitable targets for Tf-AdLac. We found that Tf-AdLac infected bFGF-stimulated FLS more efficiently than non-stimulated FLS (Figure 2).

In conclusion, we have shown that expression of hTR by FLS is increased by the addition of bFGF, and demonstrated that hTR can be applied as a target for effective gene transduction. Moreover, this type of transduction system holds promise for delivering genes to hTR-overexpressing FLS and may be used in the future for gene therapy of RA patients.

Methods

Cell Culture and Treatment

Synovial tissue samples obtained by therapeutic synovectomy from several RA patients (68.1-5.8 yr, female, N=6), who fulfilled the 1987 revised American College of Rheumatology criteria[19], were examined in the present study. All procedures involving human tissues were approved by the Ethics Committee of Kyung Hee University Medical Center, and consent forms were obtained from all patients involved in the present study. FLS were isolated by enzymatic digestion and cultured as described previously[11,20]. Confluent cells were seeded at a density of 2×10^4 cells in 24-well plates or 2×10^5 cells in 60-mm culture dishes for 24 hr, and the medium was then replaced with serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) that was supplemented with bFGF (1, 10, 100 ng/mL; Sigma, St. Louis, MO, USA).

Recombinant Adenovirus Infection

FLS were seeded at a density of 2×10^4 cells in a 24-well plate for 24 hr and then infected with a virus that carried the β-galactosidase gene in infection medium that contained 2% fetal bovine serum (FBS; Sigma) for 2 hr. After the 2-hr incubation period, the infection medium was replaced with normal growth medium that contained 10% FBS. The infected cells were incubated for 24 hr in 5% CO_2 at 37°C, and the β-galactosidase activity was assessed. The recombinant adenoviruses (AdLac, Tf-AdLac) were prepared as described previously[7].

Immunocytochemical Analysis

FLS were cultured in a 24-well plate with 12-mm glass coverslips until confluence was reached and then kept in serum-free DMEM for 2 hr. The cells were then treated with 100 ng/mL bFGF for 24 hr and fixed in 4% paraformaldehyde [PFA in 1× phosphate-buffered saline (PBS, pH 7.4; Sigma)] for 5 min

at room temperature. After permeabilization with 0.5% Triton X-100 in 1× PBS for 10 min at 4˚C, the cells were blocked for 30 min with 5% bovine serum albumin (BSA; Sigma) in PBS at 37˚C, and then exposed overnight at 4˚C to a 1 : 100 dilution of mouse anti-human CD71 (transferrin receptor; BioGenex, San Ramon, CA, USA) in PBS that contained 2% BSA. Fixed cells were incubated for 60 min at room temperature with FITC-conjugated anti-mouse IgG antibody (1 : 200 dilution; Molecular Probes, Eugene, OR, USA). The immunoreactivity for CD71 was visualized using a fluorescence microscope (Zeiss, Thornwood, NY, USA).

Western Blot Analysis

To detect transferrin receptor protein in the cell lysates, cells were cultured up to 80% confluency in 100-mm dishes and treated with bFGF or vehicle for 24 hr. The cells were harvested in lysis buffer that contained protease inhibitors and centrifuged at 12,000 rpm at 4˚C for 15 min. Thirty micrograms of the lysates were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 5% skim milk, the membranes were probed with anti-CD71 antibody and anti-β-actin antibody (Sigma) at 1 : 1,000 dilutions for 1 hr, and with peroxidase-conjugated anti-mouse IgG at 1 : 5,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hr. The immunoreactive bands were detected with Enhanced Chemiluminescence Plus (Amersham Biosciences, Little Chalfont Buckinghamshire, UK).

X-Gal Staining

FLS were fixed by incubation in a solution of 4% PFA in 1× PBS for 5 min at 4˚C. The cells were then washed twice with PBS, and a staining solution that consisted of PBS that contained 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$-$3H_2O$, 2 mM $MgCl_2$, and 1 mg/mL of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; stock dissolved at 50 mg/mL in dimethylsulfoxide) was added. Depending on the intensity of the staining, incubation at 37˚C was stopped after 1 hr.

β-galactosidase Assay

A β-galactosidase assay kit (Pierce, Rockford, IL, USA) was used to determine β-galactosidase activity according to the manufacturer's guidelines. In brief, FLS that expressed β-galactosidase were cultured in 96-well plates and lysed with the M-PER mammalian protein extraction reagent. All-in-One mammalian β-galactosidase assay reagent was added to each well. The reactions were stopped with 150 μL of stop solution provided in the kit, and the absorbance was read at 405 nm using a plate-reading spectrophotometer.

Statistical Analysis

The results are expressed as mean ± SD. The data were analyzed by one-way ANOVA followed by Newman Keul's *post hoc* analysis using the SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

Acknowledgements

We thank Dr. J Engler at the University of Alabama at Birmingham, USA for the permission of using a retargeted adenovirus. This work was partly supported by a grant from Korea Science and Engineering foundation to IJ (KOSEF R01-2003-000-11694-0).

References

1. Sutherland, R. *et al.* Ubiquitous cell-surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. *PNAS.* **78**(7):4515-4519 (1981).
2. Thomas, P., Ulf, M. L., Renate, E. G. & Steffen, G. Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. *Arthritis Res. Ther.* **2**(5):361-367 (2000).
3. Sweeney, S. E. & Firestein, G. S. Rheumatoid arthritis: regulation of synovial inflammation. *Int. J. Biochem. Cell Biol.* **36**(3):372-378 (2004).
4. Telfer, J. F. & Brock, J. H. Expression of ferritin, transferrin receptor, and non-specific resistance associated macrophage proteins 1 and 2 (Nramp1 and Nramp2) in the human rheumatoid synovium. *Ann. Rheum. Dis.* **61**(8):741-744 (2002).
5. Mohr, W., Beneke, G. & Mohing, W. Proliferation of synovial lining cells and fibroblasts. *Ann. Rheum. Dis.* **34**(3):219-224 (1975).
6. Toh, M. L. *et al.* Enhancement of Adenovirus-Mediated Gene Delivery to Rheumatoid Arthritis Synoviocytes and Synovium by Fiber Modifications: Role of Arginine-Glycine-Aspartic Acid (RGD)-and Non-RGD-Binding Integrins. *J. Immunol.* **175**(11):687-698 (2005).
7. Joung, I. *et al.* Improved gene delivery into neuroglial cells using a fiber-modified adenovirus vector. *Biochem. Biophys. Res. Commun.* **328**(4):1182-1187 (2005).
8. Bakker, A.C. *et al.* A tropism-modified adenoviral vector increased the effectiveness of gene therapy for arthritis. *Gene. Ther.* **8**(23):1785-1793 (2001).
9. Goossens, P. H. *et al.* Infection efficiency of type 5 adenoviral vectors in synovial tissue can be enhanced

with a type 16 fiber. *Arthritis Rheum.* **44**(3):570-577 (2001).
10. Schmidt, M. R., Piekos, B., Cabatingan, M. S. & Woodland, R. T. Expression of a human Coxsackie-adenovirus receptor transgene permits adenovirus infection of primary lymphocytes. *J. Immunol.* **165**(7): 4112-4119 (2000).
11. Toh, M. L. Expression of mitogen-activated protein kinase phosphatase 1, a negative regulator of the mitogen-activated protein kinases, in rheumatoid arthritis: upregulation by interleukin-1 and glucocorticoids. *Arthritis Rheum.* **50**(10):3118-3128 (2004).
12. Keystone, E. C. Strategies to control disease in rheumatoid arthritis with tumor necrosis factor antagonists-an opportunity to improve outcomes. *Nat. Clin. Pract. Rheumatol.* **2**(11):594-601 (2006).
13. Winthrop, K. L. Risk and prevention of tuberculosis and other serious opportunistic infections associated with the inhibition of tumor necrosis factor. *Nat. Clin. Pract. Rheumatol.* **2**(11):602-610 (2006).
14. Genovese, M. C. Biologic therapies in clinical development for the treatment of rheumatoid arthritis. *J. Clin. Rheumatol.* **11**(3 Suppl):S45-54 (2005).
15. Doan, Q. V., Chiou, C. F. & Dubois, R. W. Review of eight pharmacoeconomic studies of the value of biologic DMARDs (adalimumab, etanercept, and infliximab) in the management of rheumatoid arthritis. *J. Manag. Care. Pharm.* **12**(7):555-569 (2006).
16. Yeh, P. & Perricaudet, M. Advances in adenoviral vectors: from genetic engineering to their biology. *FASEB J.* **11**(8):615-623 (1997).
17. Manabe, N. *et al.* Involvement of fibroblast growth factor-2 in joint destruction of rheumatoid arthritis patients. *Rheumatology* **38**(8):714-720 (1999).
18. Wilder, R. L. *et al.* Endothelial cells and the pathogenesis of rheumatoid arthritis in humans and streptococcal cell wall arthritis in Lewis rats. *J. Cell. Biochem.* **45**(2):162-166 (1991).
19. Arnett, F. C. *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **31**(3):315-324 (1988).
20. Chabaud, M., Fossiez, F., Taupin, J. L. & Miossec, P. Enhancing effect of IL-17 on IL-1 induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J. Immunol.* **161**(1):409-414 (1998).