Freeze-thaw increases adeno-associated virus transduction of cells

Sifeng Chen,1 Clive Wasserfall,2 Matthias H. Kapturczak,1 Mark Atkinson,2 and Anupam Agarwal1

1Department of Medicine, Nephrology Research and Training Center, University of Alabama at Birmingham, Birmingham, Alabama; and 2Department of Pathology, University of Florida, Gainesville, Florida

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Chen, Sifeng, Clive Wasserfall, Matthias H. Kapturczak, Mark Atkinson, and Anupam Agarwal. Freeze-thaw increases adeno-associated virus transduction of cells. Am J Physiol Cell Physiol 291: C386–C392, 2006. First published March 1, 2006; doi:10.1152/ajpcell.00582.2005.—A combination of gene- and cell-based therapies may provide significant advantages over existing treatments in terms of their effectiveness. However, long-term efficient gene delivery has been difficult to achieve in many cell types, including endothelial cells. We developed a freeze-thaw technique which significantly increases the transduction efficiency of recombinant adeno-associated virus vectors in human aortic endothelial cells (23-fold) and in human renal proximal tubular epithelial cells (128-fold) in comparison to current methods for transduction. Freeze-thaw resulted in a transient but significant increase in cell surface area by 1.174 ± 69.8 μM² per cell. Reduction of cryogenic medium volume and repeated freeze-thaw further increased transduction efficiency by 2.8- and 2.4-fold, respectively. Trypsinization, dimethylsulfoxide, and cold temperatures, which are also involved in cell preservation, had no significant impact on transduction efficiency. Increased transduction was also observed in mesenchymal stem cells (42-fold) by the freeze-thaw method. The potential mechanism of this novel technique likely involves an increase in the net permeable area of biological membranes caused by water crystallization. These findings provide a new approach for gene delivery in various cell types, particularly in those resistant to transduction by conventional methods.

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GENE-BASED THERAPIES and cell transplantation have shown great promise as therapeutic approaches. A combination of these two strategies may bring significant reciprocal advantages. Multiple physical and viral techniques have been developed to deliver genes into cells. Physical methods, such as ultrasound (18) and electroperoration (1), as well as lipid-derived transfection reagents (24), have been used to improve gene delivery, mainly for naked DNA. The major limitation of gene delivery of naked DNA is the transient expression of the target protein. Only limited success has been achieved in virus-mediated gene delivery using physical techniques. Virus-associated transduction is usually receptor mediated and has a potential to achieve long-term gene expression (16). However, the quantity and activity of receptors varies among individual cell types, making it difficult to control for the efficiency of gene delivery (14). Long-term gene expression has been difficult to achieve in many cell types, such as endothelial cells (4), stem cells (26), and renal epithelial cells (3).

Recombinant adeno-associated viral vectors (rAAV) have several distinct advantages over other gene delivery vectors because rAAV infects cells with limited discernable side-effects (2). In addition, genes introduced by rAAV can provide continuous transgene production following a single application (12, 20). We recently reported significant transduction of endothelial cells in vitro and in vivo using alternate serotypes of rAAV1, and -5 (4). Although capsid modification and the application of alternative serotypes of viral vectors improve their transduction efficiency significantly, further improvement is required for clinical application. In this investigation, we describe a novel freeze-thaw technique developed to increase the efficiency of intracellular gene delivery.

MATERIALS AND METHODS

Construction and preparation of rAAV vectors. rAAV serotype 1 vectors expressing interleukin-10 (IL-10), green fluorescent protein (GFP), and luciferase (Luc)-enhanced yellow fluorescent protein (Luc-EYFP), respectively, as well as rAAV serotype 2 vectors expressing GFP were generated and purified by previously described methods (27).

Cell culture. Human aortic endothelial cells (HAEC) were obtained from Clonetics (Walkersville, MD) and were grown at 37°C, 5% CO2 and 95% room air in complete endothelial growth medium (EGM). EGM contains endothelial basal medium supplemented with 10% fetal bovine serum, gentamicin, amphotericin B, hydrocortisone, human epidermal growth factor (EGF), and bovine brain extract (Clonetics). These cells stain positively for endothelial specific markers, including factor VIII-related von Willebrand factor and platelet endothelial cell adhesion molecule-1 (anti-rat CD31, Antigenix America, Huntington Station, NY) and are able to uptake Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA). Rat aortic endothelial cells (RAEC) were isolated and identified as previously described (4). Cells were used for experiments at passages 5 and 6. HK-2 cells (CRL-2190), an immortalized human proximal tubule epithelial cell line from normal adult kidney, were obtained from the American Type Culture Collection (Manassas, VA) and grown in keratinocyte-serum-free medium supplemented with 5 ng/ml recombinant epidermal growth factor and 40 μg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA). The cells were grown in 100-mm tissue culture plates and studied at ~80% confluence. Primary rat mesenchymal stem cells (MSCs) were prepared from bone marrow isolated from Wistar-Furth rats based on their adherence to tissue culture surfaces as described previously (10, 19). They were grown in DMEM containing 10% fetal bovine serum. Cells at passage 3 were used for experiments.

Cell surface area measurements. HAEC (1 × 105) in 200 μl EGM with 5% DMSO were placed into one well of a flat-bottomed 96-well plate (n = 3; Corning). The cell radius before freezing was measured using an inverted microscope (model DMIRB, Leica, Bannockburn, IL) incorporated with ImagePro software (Media Cybernetics, Silver Spring, MD). Each well with cells was then placed inside a 2-ml cryogenic vial, capped, and frozen at −80°C for 6 h and in liquid nitrogen for 18 h. The cryogenic vials were then removed from liquid nitrogen. The culture wells were taken out of the cryogenic vials, thawed, placed on a 60-mm culture dish, and monitored continuously.
under the same microscope system. Cell radius (r) of 100–105 cells per time point was measured 0, 2, and 5 min after being thawed. Cell surface area was calculated as spherical surface area = 4πr².

rAAV1-vector transduction of HAEC. rAAV1-GFP, rAAV1-IL-10, or rAAV1-luciferase vectors were added to 6 × 10⁵ HAEC in 1.8 ml of complete medium with 5% DMSO at a multiplicity of infection (MOI) of 1,000 viral particles/cell, stored at −80°C for 6 h and frozen in liquid nitrogen for 16 h. Five minutes after the cells were thawed, they were washed with 10 ml complete EGM and placed in 6 wells of a 24-well plate for further culture with 5% CO₂ at 37°C. This transduction process is referred to as the freeze-thaw technique. For control, HAEC in 0.3 ml EGM were cultured to ~50% confluence in 24-well plates (~1 × 10⁵ cells/well) and infected with the same MOI of rAAV1 at 37°C for 24 h but without freeze-thaw. HAEC with the same treatment solution but without rAAV1 was used as a negative control. Cell culture medium was changed every 3 days.

To test the effect of the gene product induced by rAAV1 transduction by freeze-thaw method, cell proliferation was assessed by 3-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) assay according to the manufacturer’s protocol (22) ml was directly added to other wells after being washed with PBS. The vectors were then added to the cells at MOI of 10,000 and incubated for 24 h, as described above. To observe the effects of DMSO, cells were incubated at 4°C or 37°C in EGM with or without 5% DMSO. The effect of cold temperature (4°C) was estimated by comparing the data of cells incubated at 4°C and 37°C. For negative controls, each group was accompanied by a group with the same treatment by replacing rAAV1 solution with the same volume of PBS. For positive control, HAEC were transduced with rAAV1-luciferase using freeze-thaw technique, as described before, but at MOI of 10,000 instead of 1,000. Seven days after the treatments, the cells were harvested for measurement of luciferase activity.

rAAV1 transduction of HK-2 cells and MSCs. HK-2 cells were transduced with rAAV1-luciferase or rAAV2-luciferase at 37°C or by the freeze-thaw technique as described before at MOI of 0, 10³, 10⁴, or 10⁵. Luciferase activity was measured at day 7 after transduction. MSCs were transduced with PBS, rAAV1-GFP, or rAAV1-IL-10 at 37°C, or by the freeze-thaw technique, as described before at MOI of 10⁵. Positive GFP cells were monitored and medium IL-10 levels were detected at day 7 after transduction.

Measurement of transgene expression. GFP in cells was monitored using an inverted fluorescence microscope (model DMIRB, Leica) and pictures taken at 24 h and 1, 4, 8, and 16 wk after transduction. The number of GFP-positive cells in each field was counted with an integrated image analysis system. Medium IL-10 level was measured as described previously (8). For luciferase assay, cells were lysed in 2.5× passive lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the luciferase assay kit (Promega) and normalized to total cellular protein with the use of a protein assay (Bio-Rad Laboratories, Hercules, CA).

Measurement of cell viability and permeability. Cell viability with and without freeze-thaw was measured using trypan blue dye exclusion and the XTT assay. RAEC were slowly frozen as described above or underwent trypsinization without freeze-thaw (n = 5/group). Trypan blue uptake was assessed immediately after thawing or trypsinization. The percentage of trypan blue-stained cells was counted. The XTT assay was performed 3 days after the cells were plated (1 × 10⁴ cells/well), following either freeze-thaw or trypsinization alone, according to the manufacturer’s protocol (Promega). Lactate dehydrogenase (LDH) release was measured to evaluate cell injury and changes in cell permeability. LDH concentration in cryogenic medium of RAEC after freeze (for 24 h) and thaw was measured according to the manufacturer’s protocol (Roche Diagnostic, Indianapolis, IN) and compared with culture medium of RAEC undergoing trypsinization and maintained at 37°C for 24 h.

Statistical analysis. Results are derived from at least 3–4 independent experiments in each case. Data are represented as means ± SE. Student’s t-test, ANOVA, and Student-Newman-Keuls test were used for multiple comparisons and a value of P < 0.05 was considered significant.

RESULTS

Freeze-thaw increases cell surface area. As water freezes, its volume increases and at a cellular level, the process enlarges cells and increases their surface area. In contrast, thawing of intracellular water crystals may produce negative pressure to accelerate the entry of extracellular molecules. Because cellular conditions and medium components may impact solution and crystal volume, we measured r immediately after freezing and at 0, 2, and 5 min after thaw. We considered suspended cells as a sphere and calculated cell surface area as spherical surface area = 4πr².

HAEC radii were 25.3 ± 0.40 (before), 27.01 ± 0.43 (0 min), 25.56 ± 0.38 (2 min), and 25.32 ± 0.34 (5 min) μM (n = 100–105 cells/time point) before freeze-thaw, and at 0, 2, and 5 min after freeze-thaw, respectively. HAEC surface area immediately after freeze-thaw was 14% larger than the surface before freezing (P < 0.0001), leading to an increase of net-permeable surface area of 1,174 ± 69.8 μm² per cell. The cell surface area returned to normal within 5 min after thawing.

Time course of rAAV1 transduction using the freeze-thaw method in endothelial cells. Transduction of HAEC using the freeze-thaw technique increased GFP expression, which persisted until 16 wk (Fig. 1A). Quantitation of GFP-positive cells in the first 4 wk after transduction is shown in Fig. 1B. Interestingly, positive cells were not distributed evenly and islands of GFP-positive areas were noticed in both groups after 4 wk. At 16 wk, most areas of rAAV1-GFP-transduced wells had >95% GFP-positive cells, while this was significantly lower (<5%) in the cells transduced by the non-freeze-thaw technique (Fig. 1A).

To determine whether the cells express the transgene product continuously, HAEC were transduced with rAAV1-IL-10 using the same experimental setting as above. Secreted IL-10 levels in media supernatants were significantly increased compared with HAEC transduced with the traditional technique at 1–4 wk, respectively (Fig. 1C). To observe whether IL-10 produced from the rAAV1-IL-10 transduction with the freeze-thaw technique was functional, HAEC were studied using a cell proliferation assay. As shown in Fig. 2, the cell number in
the rAAV1-IL-10-transduced group was significantly lower than cells transduced with rAAV1-GFP or PBS. This observation confirms the functional effects of the secreted IL-10, which is known to inhibit endothelial cell proliferation (23).

To determine whether freeze-thaw-mediated gene transduction is dose dependent, HAEC were transduced with rAAV1-luciferase with or without freeze-thaw at a MOI of 1,000 and 10,000. As shown in Fig. 3A, freeze-thaw increased transduc-

![Figure 1](http://ajpcell.physiology.org/)

**Fig. 1.** Transduction of human aortic endothelial cells (HAEC) with recombinant adeno-associated virus serotype 1 (rAAV1) encoding green fluorescent protein (GFP) or IL-10. A and B: HAECs were transduced with rAAV1-GFP at a multiplicity of infection (MOI) of 1,000 viral particles/cell at 37°C (A, left panel, and B, solid line) and by the freeze-thaw technique (A, right panel, and B, dashed line). Cells were monitored for positive GFP fluorescence at the indicated time points. C: IL-10 levels in culture media from HAECs transduced with rAAV1-IL-10 (MOI = 1,000 viral particles/cell) at 37°C (open bars) and by the freeze-thaw technique (solid bars). Cell culture media (0.5 ml) were changed every first and fourth day of the week. IL-10 levels were measured by enzyme-linked immunosorbent assay (ELISA), as described in MATERIALS AND METHODS, from samples collected at the first day of every week. Values are expressed as means ± SE, n = 6 per group; P values represent significant differences using different techniques at the same time points. Each group was accompanied by a mock transduction without the viral vector. IL-10 levels of these negative controls were not detectable (data not shown).
tion efficiency by 1.1 and 23.0-fold at an MOI of 1,000 and 10,000, respectively. Luciferase activities between cells transduced at MOI of 1,000 and 10,000 without freeze-thaw were not statistically different (P > 0.05).

Effect of rAAV1 concentration on transduction. According to the impact of freeze-thaw on cell surface, the increase of permeable cell surface is temporary. Thus higher extracellular rAAV1 concentration should significantly increase intracellular entry of the vector. 6 × 10⁵ HAEC were suspended in 0.25, 1.0, and 1.8 ml medium, respectively, and transduced with the same amount of vectors (MOI = 1,000) using the freeze-thaw technique. HAEC in 0.25 ml medium had significantly higher gene expression than HAEC suspended in 1.0 and 1.8 ml medium, whereas gene expression in HAEC suspended in 1.0- and 1.8-ml media was not significantly different (Fig. 3).

Effect of dual freeze-thaw on rAAV1 transduction. If the permeable area of cell membrane increased by freeze-thaw is temporary and the increase results in rAAV1 transportation across the membrane, then repeating the process of freeze-thaw should further increase rAAV1 transduction. Our result showed that gene expression in HAEC that underwent freeze-thaw twice in the presence of rAAV1 was further increased by 2.4 times when compared with that of HAEC undergoing freeze-thaw only once (Fig. 3C).

Effects of trypsinization, low temperature, and DMSO on rAAV1 transduction. Because cell preservation by liquid nitrogen involves trypsinization, low temperature, and DMSO, their effects on rAAV1 transduction were also evaluated. As shown in Fig. 4, trypsinization, low temperature, and DMSO had no impact on rAAV1 transduction. As observed previously, freeze-thaw increased the transduction significantly.

Freeze-thaw-mediated rAAV transduction in HK-2 cells and rat mesenchymal stem cells. To test whether the freeze-thaw technique can be applied to transduction of other cells, HK-2
and mesenchymal stem cells (MSCs) were transduced with rAAV1 vectors. The result showed that no luciferase expression was detected in HK-2 when transduced with rAAV1-luciferase at 37°C 7 days after transduction. Freeze-thaw technique increased luciferase expression by 128-fold (Fig. 5A).

rAAV-luciferase serotype 2 mediated modest luciferase transduction in HK-2 cells at 37°C. However, the transduction efficiency at 37°C was saturated when MOI was $10^4$. Further increase of MOI did not increase transduction. Freeze-thaw technique did not result in higher luciferase expression at MOI of $10^3$. Interestingly, freeze-thaw technique increased rAAV2-mediated luciferase expression by 42% and nine-fold at MOI of $10^4$ and $10^5$, respectively (Fig. 5B).

Freeze-thaw transduction of MSCs with rAAV1-GFP resulted in 60% GFP-positive cells (Fig. 5C) and increased IL-10 levels in the media by 42-fold after transduction with rAAV1-IL-10 (Fig. 5D). No GFP-positive cells were detected in MSCs transduced with PBS, rAAV1-GFP, and rAAV-IL-10 (MOI = $10^4$), respectively at 37°C.

**Effects of freeze-thaw on cell viability and permeability.** Cell viability measured by trypan blue dye exclusion showed that there was a trend toward more trypan blue-positive cells in the freeze-thaw group (16.92 ± 2.17%) compared with the cells undergoing trypsinization alone (8.94 ± 2.87%). However, this difference was not statistically significant ($P = 0.06$). Cell viability by the XTT assay showed no significant differences in the cells undergoing freeze-thaw compared with the non-freeze-thaw group (1.377 ± 0.13 and 1.153 ± 0.07 optical densitometric units, respectively, $P = 0.14$). These results suggest that the freeze-thaw procedure does not significantly affect cell viability. LDH release from cells after freeze and thaw was measured and compared with cells undergoing trypsinization and maintained at 37°C for 24 h. The release of LDH from cells after freeze-thaw was similar to the spontaneous release at 24 h in the non-freeze-thaw group (0.38 ± 0.07 and 0.39 ± 0.01 optical densitometric units, respectively, $P = 0.92$). There was no detectable LDH release before cells were subjected to freezing.

**DISCUSSION**

The major components of cell membranes are both hydrophilic and hydrophobic. The overall balance of interactions of all components decides the proximity of membrane and intracellular constituents. In normal conditions, the cell membrane is permeable only to water and some small gaseous molecules,
such as CO₂, nitric oxide, CO, and O₂. Most electrolytes and large molecules pass cell membranes through specific transporters and receptors. Cryopreservation of both virus and cells in liquid nitrogen is usually necessary in laboratory research and cell therapy (e.g., cell transplantation). In accordance with the temperature-volume curve of water, we expect intracellular volume to increase during freezing. To prove our estimation, HAEC radius was measured before and after freeze-thaw. The result showed that HAEC radius increased by 6.8%, resulting in a net-permeable surface area increase of 1,174 ± 69.8 μm² (14%). The increase of cell radius was temporary. It returned to normal in 5 min. The quick rescaling of permeable holes may prevent vectors from reversely diffusing out of the cells. Freeze-thaw has been reported to be able to increase intracellular entry of electrolytes (13). Considering the significant increase of net permeable area of cell surface after freeze-thaw, we expected that it might also promote intracellular entry of macromolecules, including viral vectors. Our hypothesis was confirmed by transduction of HAEC that otherwise demonstrate low transduction efficiency with a nonsecretory reporter gene product, GFP, and a secreted gene product, IL-10, using rAAV1 as a viral gene delivery system. The increased gene expression resulting from the freeze-thaw technique persisted at least 16 wk in HAEC.

Because freeze-thaw-mediated increases in cell surface permeable area is temporary, the viral vector has only limited time to enter the cells. If this is the case, increase of extracellular vector concentration without increasing MOI should accelerate vector entry. We found that freeze-thaw-mediated transduction efficiency of HAEC in 0.25 ml was 277% higher than that in 1.8 ml. This feature is very useful because 1.8 ml is the minimum volume to cover 6 × 10⁵ HAEC with ~50% confluence as well as other adherent cells. According to our hypothesis, membrane permeability will increase again following freeze-thaw after permeability returns to normal after the initial freeze-thaw. To confirm this expectation, HAEC suspension containing rAAV1-luciferase was frozen and thawed twice. It was found that luciferase expression in HAEC undergoing freeze-thaw twice was 3.4 times that of HAEC being freeze-thawed only once. Because the second freeze-thaw increased luciferase expression >100%, it cannot be explained merely by increased cell surface permeability. It is possible that when the cells were frozen, the biological membranes of nuclei and intracellular organelles were also increased and the second freeze-thaw increased viral particles that had already entered the cells due to the first freeze-thaw to enter nuclei and other organelles. We also found that increasing MOI from 1,000 to 10,000 resulted in a 14.3-fold increase in gene expression when the cells were transduced using freeze-thaw technique, whereas the change of gene expression was insignificant when the cells were transduced at 37°C without freeze-thaw. The latter may be due to the saturation of AAV receptors. Our hypothesis was further corroborated in other cell types, including HK-2 and MSC.

Because freeze-thaw resulted in temporary increases in cell surface permeable area, a potential contribution of altered cell viability was evaluated. The results showed that there was a trend toward more trypan blue positive cells in the freeze-thaw group. However, the XTT assay did not show any difference between cells undergoing freeze-thaw compared with trypsinization alone. This result suggests that the freeze-thaw procedure does not adversely affect cell viability and is therefore not a major factor contributing to the higher transduction efficiency observed in cells undergoing freeze-thaw.

As described in a recent review (6), rAAV1 transduction includes seven stages: 1) viral binding to receptor/coreceptor, 2) endocytosis, 3) intracellular trafficking through the endosomal compartment, 4) endosomal escape, 5) nuclear import, 6) viron uncoating, and 7) viral genome conversion. On the basis of our data, we speculate that freeze-thaw-mediated rAAV1 transduction may bypass stages 1–4 increasing thereby transduction efficiency. However, it is possible that other mechanisms may contribute to the observed increase in transduction. Because it is a single-stranded DNA virus, AAV requires second-strand synthesis to enable transgene expression (7). Chemical and physical stresses have been shown to stimulate AAV transduction through several mechanisms including acceleration of second strand synthesis, nuclear transport, uncoating, intracellular viral processing, and receptor expression (4, 7, 9, 21, 25, 26). The process of freeze-thaw is clearly a form of physical stress for cells and may therefore enhance transduction by one or more of these processes.

HAEC and MSCs were used as target cells to evaluate the freeze-thaw technique because their great potential in cell therapy of cardiovascular diseases and organ transplantation (5, 11). Limited advances have been made in achieving successful long-term gene transfer in many cell types, including endothelial cells and stem cells. For example, current gene delivery methods in endothelial cells using viral and nonviral vectors has several drawbacks, including transient and low transduction efficiency, and adverse immune effects of some of the viral vectors (15, 17). More consistent and reliable techniques will significantly improve gene-based cell therapy.

Our results are directly relevant to genetic engineering of multiple therapeutic cells. Transduced gene copies are usually diluted along with the proliferation of transfused cells in the recipients. Thus our technique cannot only be used for the transduction of resistant cells but also to increase transduced gene copies in nonresistant cells. The freeze-thaw technique may also be applicable to deliver RNA, naked DNA, and proteins into cells. In conclusion, this ex vivo gene delivery technique can be very useful in engineering cells for cell therapy and will have several clinical applications.

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freezing and thawing increases gene transduction


