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High-level Elevation of Transgene Expression *via* the Joint Effect of Trichostatin A and D-glucose in a Malignant Pleural Mesothelioma-derived Cell Line Resistant to Adenovirus Vector-based Transgene Expression

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Abstract

Objective: Malignant Pleural Mesothelioma (MPM), a locally invasive tumor, is treated with a combination of surgical, radiation, and medical therapies, but the treatment efficacy remains insufficient. Gene therapy for MPM has been attempted to improve the prognosis of the disease, but has not yet reached a practical level. One of the barriers to MPM gene therapy is the resistance of MPM cells to transgene expression. Therefore, the purpose of this study was to find techniques to achieve high-level transgene expression in MPM cells resistant to transgene expression.

Methods: We evaluated two MPM cell lines, NCI-H28 (H28) and NCI-H2052 (H2052), for their resistance to transgene expression, and then examined whether transgene expression in the resistant H2052 cells was enhanced by treating the cells with D-glucose, an extracellular signal-regulated kinase 1/2 inhibitor LY3214996 (LY), and a histone deacetylase inhibitor trichostatin A (TSA), which have been reported to increase transgene expression. Cellular transgene expression was evaluated by a reporter gene assay in which a human cytomegalovirus immediate early (CMV) promoter-controlled Enhanced Green Fluorescent Protein (EGFP) gene was inserted into the cells using a human adenovirus (ADV) vector. The extent of EGFP gene expression was examined by fluorometric assay, fluorescence microscopy, and EGFP quantification by ELISA.

Results: The fluorescence intensity in H2052 cells was only 13.9% of that in H28 cells. D-glucose treatment of H2052 cells after transduction (post-treatment) increased the fluorescence intensity in H2052 cells by only 1.9-fold. LY treatment of H2052 cells before transduction (pretreatment) increased the fluorescence intensity in the cells by only 1.7-fold. TSA pretreatment increased the fluorescence intensity in H2052 cells by as much as 6.9-fold, to almost the same level as that in H28 cells. When the TSA-pretreated H2052 cells were posttreated with D-glucose, the fluorescence intensity in H2052 cells was enhanced to 400% of that in H28 cells. The elevated EGFP gene expression by the joint effect of TSA and D-glucose was also confirmed by fluorescence microscopy and EGFP quantification by ELISA.

Conclusion: It was suggested that TSA pretreatment could remove the resistance to transgene expression of MPM cells and the joint effect of TSA and D-glucose could highly enhance transgene expression in the resistant MPM cells.

Keywords: Malignant pleural mesothelioma • Transgene expression • Human adenovirus vector • D-glucose • Trichostatin A

Introduction

Malignant Pleural Mesothelioma (MPM) is a locally invasive tumor [1]. Most cases of MPM are not detected until they reach advanced stages and the survival period remains only 12 to 36 months. Combinations of surgery, radiation, and chemotherapy have been applied to treat MPM but the treatment effects remain insufficient. The limitation in the existing treatments has drawn attention to gene therapy as a possible means of improving MPM prognosis. Many different gene therapy trials have been conducted for MPM [2-5] but the original goal of fully curative therapies remains elusive.

The main reason why the effects of cancer gene therapy remain impractical is that the foreign genes used for cancer treatment are not expressed at high enough levels. Because it is essential to express therapeutic foreign genes in target cancer cells sufficiently to treat the cancer, it is important to ensure sufficient transgene expression even in cancer cells that are resistant to transgene expression. Moreover, increased expression of foreign genes is expected to reduce the number of viral vectors to be administered. *In vivo* administration of viral vectors induces neutralizing antibodies against the vectors and the ant vector antibodies can inhibit transgene expressions [6,7]. The reduction in viral vector dosage contributes to preventing the ant vector antibodies from hindering viral vector-based gene introduction by alleviating the increase in the ant vector antibodies.

Several causes of low transgene expression in cancer cells have been reported. First, when using non-viral vectors to introduce foreign genes into cells, the efficiency of gene transfer is basically low [8]. The low efficiency of foreign gene introduction leads to low expression of the gene. Despite continuous efforts to improve this problem, the results have not been satisfactory. On the contrary, when using viral vectors to introduce foreign genes into cells, we can achieve much higher expression of the genes than

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when using non-viral vectors. Therefore, in this study, we selected an MPM cell line that exhibits very low transgene expression even when the foreign gene is introduced into the MPM cell line using a human adenovirus type 5 (ADV) vector, and searched for a technology to increase transgene expression in this cell line. We previously reported that D-glucose enhanced the transgene expression in monkey kidney-derived CV-1 cells into which a foreign gene was introduced using a non-viral vector [9]. We therefore conjectured that D-glucose might effectively enhance transgene expression in MPM cells that were resistant to ADV vector-based transgene expression.

Secondly, in cases where the target cancer cells do not have enough virus receptors, even if a virus vector is used for introduction of foreign genes, the efficiency of the gene introduction is low, resulting in low expression of the gene. For instance, it has been shown that a deficiency of Coxsackievirus and Adenovirus Receptors (CAR) on tumor cells restricts the efficacy of ADV vectors in TP53 gene replacement therapy and herpes simplex virus thymidine kinase/ganciclovir suicide gene therapy [10]. It is thus necessary to increase CAR production in the tumor cells in order to potentiate these gene replacement therapies. Liu J, et al. reported that inhibition of an extracellular signal-regulated kinase 1/2 (ERK1/2) in mouse cardiac stem cells prior to transduction of a reporter gene using the ADV vector system increased the number of CAR on the cell surface and upregulated the expression of the reporter gene in the cells [11]. Treatment of cancer cells with an ERK1/2 inhibitor is expected to enhance transgene expression in the MPM cells resistant to transgene expression.

Thirdly, heterogeneity of cancer cells often presents a major challenge, and MPM is no exception. Indeed, MPM cells have both inter- and intra-cancer heterogeneity [12]. There must also be heterogeneity in the permissiveness of MPM cells to transgene expression, which raises concerns that some MPM cells may have suppressed transgene expression in the cells. Histone Deacetylase (HDAC) inhibitors have been reported to enhance transgene expression in various cell types [13-16]. Bishop CL, et al. [17] reported that an HDAC inhibitor could activate virus-like particle-delivered transgenes that had been silenced by the cellular defense systems in mouse Swiss albino 3T3 and cos7 cells. HDAC inhibitors thus may enhance transgene expression not only in MPM cells sensitive to but also in MPM cells resistant to transgene expression.

Collectively, the above findings led us to examine whether ADV vector-based transgene expression in an MPM cell line resistant to transgene expression could be elevated by treatment with D-glucose, an ERK inhibitor, and/or an HDAC inhibitor.

Materials and Methods

Reagents

D-glucose and Trichostatin A (TSA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). LY3214996 (LY) was obtained from Selleck Biotechnology (Tokyo).

Cell lines and cell culture

The human embryonic kidney-derived cell line, 293AD, was purchased from Cell Biolabs (San Diego, CA) and used for preparation of a recombinant ADV vector, Ad-CMV-GFP (SignaGen Laboratories, Rockville, MD). The 293AD cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo) containing 0.3 mg/ml of L-glutamine, 50 μg/ml of kanamycin, 0.45% D-glucose, and 10% fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA) at 37 °C under 5% CO_2 in air and maintained in the same medium but containing 5% FBS after Ad-CMV-GFP adsorption. Human MPM-derived cell lines, NCI-H28 (H28) and NCI-H2052 (H2052), were obtained from the American Type Culture Collection (Manassas, VA) and used to examine the enhancing effects of D-glucose, LY, and TSA on transgene expression. Both cell lines were grown in RPMI1640 (RPMI; Nissui Pharmaceutical) containing 0.3 mg/ml of L-glutamine, 50 μg/ml of kanamycin, 0.2% D-glucose, and 10% FBS at 37 °C in a 5% CO_2 atmosphere and maintained in the same medium but containing 5% FBS after ADV vector-mediated transduction.

Preparation of Ad-CMV-GFP

Ad-CMV-GFP is a replication-defective ADV vector Expressing Enhanced Green Fluorescent Protein (EGFP) under the control of a human Cytomegalovirus immediate early (CMV) promoter in mammalian cells. We propagated Ad-CMV-GFP in 293AD cells at a multiplicity of infection of more than 5 Plaque-Forming units/cell (PFU) and purified the ADV vector by a simple method using discontinuous CsCl gradients [18]. The Ad-CMV-GFP-infected 293AD cells were disrupted by three freeze-thaw cycles, followed by low-speed centrifugation (1,600 xg, 15 min, 4 °C) of the disrupted cell solution to remove large cell debris. Then the supernatant was subjected to concentration and purification of Ad-CMV-GFP. In brief, the supernatant was overlaid on the first CsCl discontinuous gradient, which was made up of 5 ml of 2.2 M CsCl in 10 mM Tris-HCl (pH 8.0) and 5 ml of 4 M CsCl in 10 mM Tris-HCl (pH 8.0), and then centrifuged at 15,670 xg for 15 h at 6 °C. The visible band consisting of Ad-CMV-GFP particles that gathered at the interface between 4.0 M and 2.2 M CsCl layers in the first centrifugation was collected and mixed with an equal volume of 6.5 M CsCl in 10 mM Tris-HCl (pH 8.0). This mixture was transferred to a new centrifuge tube, and 4 ml of 4.0 M CsCl in 10 mM Tris-HCl (pH 8.0), 4 ml of 2.2 M CsCl in 10 mM Tris-HCl (pH 8.0), and 14 ml of 0 M CsCl in 10 mM Tris-HCl (pH 8.0) was overlaid on the mixture in this order. Then, the tube was centrifuged at 15,670 xg for 22 h at 6 °C. Ad-CMV-GFP migrated to the interface between the 4.0 M and 2.2 M CsCl layers in the second centrifugation and formed a sharp band. The band containing the ADV vector was recovered and the ADV vector solution was dialyzed against a solution containing 10 mM Tris·HCl (pH 8.0) and 10% glycerol to remove CsCl from the solution. After dialysis, the purified Ad-CMV-GFP solution was divided into aliquots and stored at -80°C until use. The infectious titer of the purified Ad-CMV-GFP solution was determined by a 50% tissue culture infectious dose ($TCID_{50}$) assay [19] using 293AD cells, calculated as $TCID_{50}$/ml according to the Reed and Muench method [20] and converted to a value in PFU/ml [21]. After thawing, the cryopreserved Ad-CMV-GFP solution was diluted with serum-free DMEM for 293AD cells and serum-free RPMI for H28 and H2052 cells, and then used for the experiments.

Reporter gene assay

H28 and H2052 cells were plated at 1.6×10^5 cells per ml. The volumes of the cell suspension added to each well of a 96-well microplate, 6-cm dish, and 25-cm^2 flask were 0.125 ml, 5 ml, and 6 mL, respectively. Twenty-four hours after seeding, the cells were inoculated with Ad-CMV-GFP at an MOI equivalent to 100 PFU/cell and incubated for adsorption for 2 h at 37 °C in a 5% CO_2 atmosphere. The inoculum was removed from each culture after adsorption and the cells were rinsed with serum-free RPMI. Then, the cells were further cultured in the maintenance medium supplemented with the indicated concentrations of each reagent. The volume of the maintenance medium added at this time was double the volume at the time of cell seeding. At 48 h after transduction, we analyzed the cells by fluorometry, fluorescence microscopy, and quantification of EGFP by an enzyme-linked immunosorbent assay (ELISA).

Fluorometry

At 48 h after transduction, the culture medium of the transduced cells in the 96-well microplate was removed and the cells were lysed with 200 μl/well of lysis buffer {25 mM Tris–HCl (pH 7.8), 2 mM EDTA, 10% glycerol, and 0.1% Triton X-100}. EGFP expression was then quantified using a fluorescent microplate reader (Fluoroskan Ascent FL; Thermo Bio Analysis, Tokyo) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

After subtracting the value of the mock-transduced cells from that in the transduced cells as background, we evaluated all fluorescence intensities as a percentage of that of H28 cells untreated with any reagent.

Microscopic observation

At 48 h after transduction, we replaced the culture medium of the transduced cells in a 25-cm^2 flask with 3 ml of Dulbecco's phosphate buffered saline, observed EGFP-expressing cells directly under a CKX53-21PH cell culture microscope with a CKX3-RFA fluorescence illuminator (Olympus,

Tokyo), and photographed them with an HD Lite digital camera (RelyOn, Tokyo).

Quantification of EGFP and PML protein

For EGFP quantification, we prepared a lysate from the transduced cells in a 6-cm dish at 48 h after transduction and measured its EGFP level using a GFP ELISA Kit (Abcam Plc, Cambridge, UK) according to the manufacturer's instruction. The protein concentration of the cell lysate was determined using a Pierce BCA Protein Assay Kit (Life Technologies Japan, Tokyo) according to the manufacturer's instructions. For Promyelocytic Leukemia (PML) protein quantification, we prepared H28 and H2052 cells in a 96-well microplate as described above. After incubating the cells for 48 h in the presence or absence of 1 μM TSA, we measured PML protein levels using a PML Colorimetric Cell-Based ELISA Kit (Assay Biotechnology Company, Fremont, CA). We normalized the PML protein levels using the glyceraldehyde-3-phosphate dehydrogenase levels according to the manufacturer's instructions.

Statistical analyses

Statistically significant differences between groups were determined by Student's *t*-test. A p-value less than 0.05 was considered significant.

Results

Strong resistance of H2052 cells to ADV vector-based transgene expression and ineffective enhancement by D-glucose of transgene expression in H2052 cells. Two MPM-derived cell lines, H28 and H2052, were transduced with a CMV promoter-controlled EGFP gene by using an ADV vector, and fluorescence intensities in both cell lines were assayed at 48 h after transduction as described in the Materials and Methods (Figure 1). Although H28 and H2052 cells were prepared, transduced with the reporter gene, and fluorometrically assayed under the same condition, H2052 cells showed only 13.9% of the fluorescence intensity in H28 cells, indicating apparent resistance of H2052 cells to transgene expression.

We previously reported that D-glucose increased transgenic expression of an EGFP gene that was introduced into mammalian cells using a cationic polymer-based vector [9]. Namely, incubating mammalian cells in the presence of a relatively high concentration of D-glucose after transfection with an FGFP gene resulted in augmented fluorescence intensity in the cells with increased transcription and translation of the reporter gene. We tested whether this enhancing effect of D-glucose could also be used for transgene expression in ADV vector-transduced H2052 cells.

H28 and H2052 cells were transduced and incubated in the medium additionally supplemented with 0%, 1%, 2%, 3%, 4%, 5%, or 6% D-glucose until 48 h after transduction. Then, we assayed both cells fluorometrically in the same way as described above (Figure 2). The fluorescence intensity in H2052 cells was increased in a dose-dependent manner by the addition of D-glucose to the culture medium after transduction (posttreatment), with an additional D-glucose concentration of 4% yielding the maximum fluorescence intensity, i.e., 1.9-fold greater than in the H2052 cells without D-glucose treatment (Figure 2B). However, the maximum fluorescence intensity obtained by treating H2052 cells with 4% D-glucose was only 20.6% of that in the D-glucose-untreated H28 cells. Thus, we have no choice but to conclude that the enhancing effect of D-glucose on transgene expression in H2052 cells is not sufficient for practical use.

Fluorescence intensity in H28 cells also increased in a dose-dependent manner by the addition of D-glucose to the culture medium. Treatment with additional 6% D-glucose resulted in maximum fluorescence intensity in H28 cells, which was 311% of that in the D-glucose-untreated H28 cells. The result that D-glucose treatment sufficiently increased the fluorescence intensity in H28 cells would seem to rule out the possibility that all MPM cells are resistant to the D-glucose-induced enhancement of transgene expression, and suggests that resistance is an obstacle to the enhancing effects of D-glucose. We must therefore find a way to overcome this resistance of H2052 cells to transgene expression.

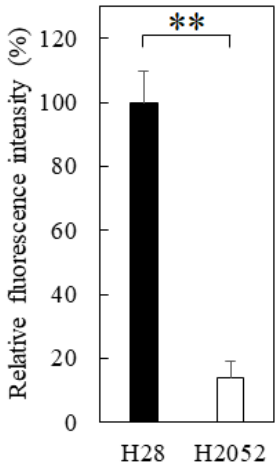

Figure 1. Strong resistance of H2052 cells to transgenic expression of an EGFP gene delivered by an ADV vector. H28 and H2052 cells were transduced with a CMV promoter-controlled EGFP gene by using an ADV vector, and fluorescence intensity was measured at 48 h after transduction as described in the Materials and Methods. Fluorescence intensities in H28 and H2052 cells were evaluated as a percentage of that in H28 cells. Columns and error bars represent the means of the quadruplicate experiments and their standard deviations, respectively. Black and white columns show data for H28 and H2052 cells, respectively. A double asterisk indicates $p<0.01$.

Enhancing effects of LY and TSA on ADV vector-based transgene expression in H28 and H2052 cells

It has been reported that the major reason why cells become resistant to ADV vector-based transgene expression is a shortage of cellular ADV receptors, CAR [10] and that CAR are increased by inhibiting ERK1/2 [11]. Therefore, we examined whether an ERK1/2 inhibitor, LY, could greatly improve transgenic expression of the ADV vector-delivered EGFP gene in H2052 cells. H28 and H2052 cells were pretreated with 0, 0.15, 0.5, 1.5, 5, 15, and 50 μM LY for 48 h, then transduced, and assayed fluorometrically as described above (Figure 3). Pretreatment of H2052 cells with 50 μM LY increased the fluorescence intensity in the cells from 40.0% to 68.7% of that in the LY-untreated H28 cells (Figure 3B). That is, the maximum increase in fluorescence intensity by LY pretreatment of H2052 cells was only 1.7-fold. These results suggested that LY pretreatment of H2052 cells could not remarkably improve transgene expression in H2052 cells which were transduced with the ADV vector system.

Pretreatment of H28 cells with 50 μM LY also increased fluorescence intensity in the cells by only 1.6-fold, like that in H2052 cells (Figure 3A).

The fluorescence intensity in the LY-untreated H2052 cells in this experiment was 40.0% of that in the LY-untreated H28 cells, which was higher than the results in Figures 1 and 2. This may be because the fluorescence intensity in this experiment tended to be relatively high overall.

TSA was previously shown to activate a silenced foreign gene in mammalian cells resistant to transgene expression [17]. Therefore, we tested whether pretreatment of H2052 cells with an HDAC inhibitor, TSA, could increase transgene expression in H2052 cells (Figure 4). H28 and H2052 cells were pretreated with 0, 0.03, 0.1, 0.3, 1, and 3 μM TSA for 48 h, transduced, and assayed fluorometrically as described above. TSA increased the fluorescence intensity in H2052 cells in a concentration-dependent manner (Figure 4B). Pretreatment of H2052 cells with 1 μM TSA increased the fluorescence intensity in the cells from 13.7% to 94.3% of that of the TSA-untreated H28 cells. That is, TSA pretreatment increased the fluorescence intensity in H2052 cells by as much as 6.8 times. The result that the fluorescence intensity in the H2052 cells could be magnified by pretreatment of the cells with 1 μM TSA to almost the same level as that of the TSA-untreated H28 cells suggests that the resistance of H2052 cells to transgene expression was abolished by TSA. TSA pretreatment did not significantly increase fluorescence intensity in H28 cells which were permissive for transgene expression (Figure 4A), suggesting that TSA does not have a significant effect on cells that are not resistant to transgene expression.

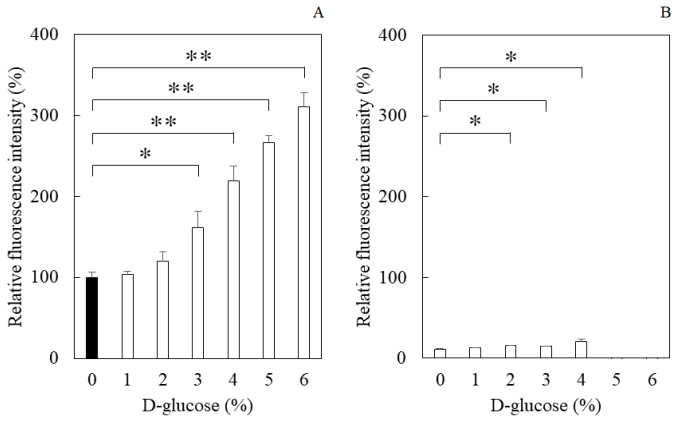

Figure 2. Enhancing effects of D-glucose on transgenic expression of an ADV vector-delivered EGFP gene in H28 and H2052 cells. H28 **(A)** and H2052 **(B)** cells were transduced with a CMV promoter-controlled EGFP gene by using an ADV vector, incubated in the medium additionally supplemented with 0%, 1%, 2%, 3%, 4%, 5%, or 6% D-glucose until 48 h post-transduction and then assayed for fluorescence intensity as described in the Materials and Methods. Fluorescence intensities in H28 and H2052 cells were evaluated as a percentage of that in the D-glucose-untreated H28 cells. Columns and error bars represent the means of the triplicate experiments and their standard deviations, respectively. A black column shows data for the D-glucose-untreated H28 cells and white columns show other data. Single and double asterisks indicate $p<0.05$ and $p<0.01$, respectively.

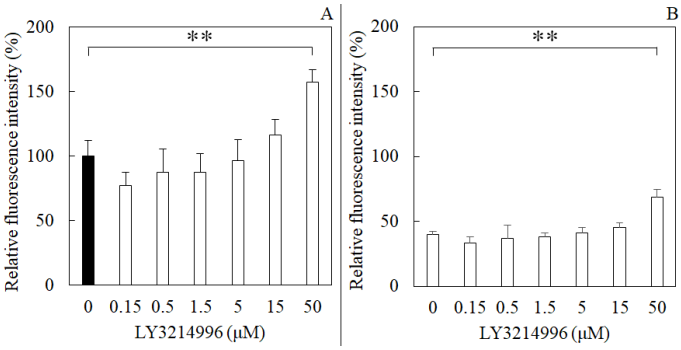

Figure 3. Enhancing effects of LY on transgenic expression of an ADV vector-delivered EGFP gene in H28 and H2052 cells. H28 **(A)** and H2052 **(B)** cells were treated with 0, 0.15, 0.5, 1.5, 5, 15, or 50 μM LY for 48 h, and then transduced with a CMV promoter-controlled EGFP gene by using an ADV vector. At 48 h after transduction, the cells were assayed for fluorescence intensity as described in the Materials and Methods. Fluorescence intensities in H28 and H2052 cells were evaluated as a percentage of that in the LY-untreated H28 cells. Columns and error bars represent the means of the quadruplicate experiments and their standard deviations, respectively. A black column shows data for the LY-untreated H28 cells and white columns show other data. Double asterisks indicate $p<0.01$.

Enhancing effect of D-glucose on transgene expression in the TSA-pretreated H2052 cells

H2052 cells pretreated with 1 μM TSA no longer appear to be resistant to transgene expression (Figure 4). If the TSA-pretreated H2052 cells were sensitive to transgene expression, it would be expected that D-glucose would also exhibit its enhancing effects on transgene expression in the cells. To confirm this expectation, we fluorometrically analyzed the stimulatory effect of elevated D-glucose on transgene expression in the 1 μM TSA-pretreated H2052 cells (Figure 5). As expected, D-glucose additional supplementation increased the fluorescence intensity in the TSA-pretreated H2052 cells in a dose-dependent manner. At the additional D-glucose concentration of 4%, the fluorescence intensity in the TSA-pretreated H2052 cells was increased to 400% of that in H28 cells without either TSA or D-glucose treatment. These results suggest that the combination of TSA-pretreatment and D-glucose-posttreatment induced a clear increase in transgene expression in H2052 cells (Figures 4 and 5).

Unfortunately, however, when the TSA-pretreated H2052 cells were cultured in the medium additionally supplemented with 3% or 4% D-glucose, severe damages such as cell rounding and atrophy were observed (data not shown).

The cooperative effect of TSA and D-glucose revealed through fluorescence analysis was confirmed by fluorescence microscopic observation (Figures 6A-6D) and quantification of EGFP levels in the cells (Figure 6E). Severe cell damage was observed when the TSA-pretreated H2052 cells were posttreated with D-glucose at an additional concentration of 3% or more. To avoid this cytotoxicity, we posttreated H2052 cells with additional 2% D-glucose. The signal of H2052 cells not treated with TSA or D-glucose (Figure 6B) was clearly weaker than that of H28 cells not treated with TSA or D-glucose (Figure 6A), whereas the signal of H2052 cells pretreated with TSA (Figure 6C) was comparable to that of the untreated H28 cells. The signal of H2052 cells pretreated with TSA and posttreated with D-glucose (Figure 6D) was clearly stronger than that under any other conditions.

The amount of EGFP in H2052 cells not treated with TSA or D-glucose was only 15.7% of that in H28 cells not treated with TSA or D-glucose. TSA pretreatment increased the amount of EGFP in H2052 cells to 125.2%—and the combined treatment with TSA and D-glucose further increased it to 216.9%—of that in the untreated H28 cells (Figure 6E). These results confirmed the joint effect of TSA and D-glucose on transgene expression in H2052 cells (Figures 6A-6E).

As mentioned above, TSA pretreatment abolished the resistance of H2052 cells to transgene expression (Figure 4B). It has been reported that TSA suppresses cytokine-induced increases in PML protein [22] and inhibits transcriptional repression mediated by PML protein [23]. If the resistance of H2052 cells to transgene expression observed in our experiment was due to PML protein-mediated transcriptional repression, we would expect the amount of PML protein in the TSA-untreated H2052 cells to be greater than that in the TSA-untreated H28 cells, and we would expect the amount of PML protein in H2052 cells to be reduced by TSA treatment. We therefore used an ELISA to quantify the PML protein levels in H2052 cells treated with 1 μM TSA for 48 h and in H28 and H2052 cells without TSA treatment, as described in the Materials and Methods (Figure 7). The results showed that the amount of PML protein in the TSA-untreated H2052 cells was significantly lower than that in the TSA-untreated H28 cells, and the amount of PML protein in H2052 cells was significantly increased by treatment with 1 μM TSA. It was therefore suggested that the resistance of H2052 cells to transgene expression is not mediated by the PML protein.

Discussion

Due to the limitations of existing treatments for MPM, gene therapy for MPM is currently under development [2-4]. For such gene therapy to be effective, foreign genes must be sufficiently expressed in target MPM cells.

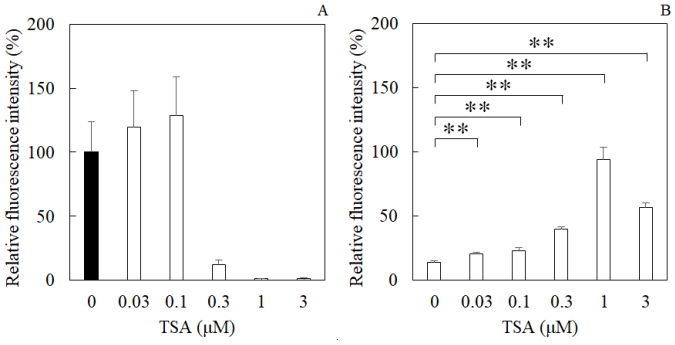

Figure 4. Enhancing effects of TSA on transgenic expression of an ADV vector-delivered EGFP gene in H28 and H2052 cells. H28 **(A)** and H2052 **(B)** cells were pretreated with 0, 0.03, 0.1, 0.3, 1, or 3 μM TSA for 48 h, then transduced with a CMV promoter-controlled EGFP gene by using an ADV vector. At 48 h after transduction, the fluorescence intensity in the cells was as described in the Materials and Methods. Fluorescence intensities in H28 and H2052 cells were evaluated as a percentage of that in the TSA-untreated H28 cells. Columns and error bars represent the means of the quadruplicate experiments and their standard deviations, respectively. A black column shows data for the TSA-untreated H28 cells and white columns show other data. Double asterisks indicate $p<0.01$.

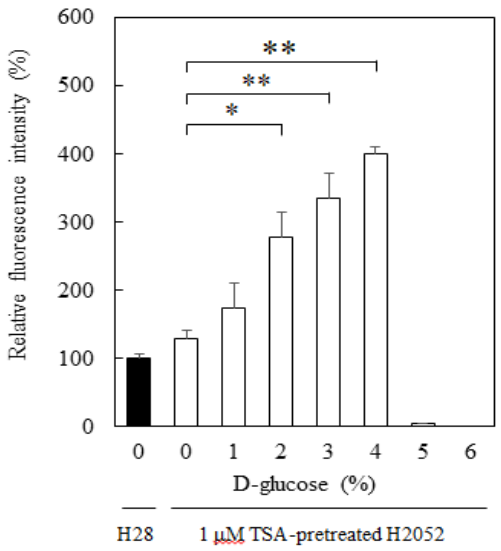

Figure 5. Enhancing effects of D-glucose on transgenic expression of an ADV vector-delivered EGFP gene in the TSA-pretreated H2052 cells. We examined whether a combination treatment with TSA and D-glucose would realize more effective transgene expression in H2052 cells, an MPM cell line resistant to transgene expression. As described in the Materials and Methods, H2052 cells were pretreated with 1 μM TSA for 48 h, and then the pretreated H2052 and the TSA-untreated H28 cells were transduced with a CMV promoter-controlled EGFP gene using an ADV vector. Then, 0%, 1%, 2%, 3%, 4%, 5%, or 6% D-glucose was additionally supplemented to the medium and the cells were incubated until 48 h after transduction and assayed for their fluorescence intensities. Fluorescence intensities in H28 and H2052 cells were evaluated as a percentage of that in the H28 cells treated with neither TSA nor additional D-glucose. Columns and error bars represent the means of the triplicate experiments and their standard deviations, respectively. A black column shows data for H28 cells not treated with TSA or D-glucose and white columns show data for H2052 cells. A single asterisk and double asterisks indicate $p<0.05$ and $p<0.01$, respectively.

However, MPM cells exhibit inter- and intra-cancer heterogeneity in various characteristics [12] including in their permissiveness to transgene expression. There is thus concern that gene therapy may not be sufficiently effective in MPM cells that have low permissiveness—i.e., resistance—to transgene expression. To dispel this concern, there is an urgent need to develop methods to ensure the transgene expression necessary for gene therapy in MPM cells resistant to transgene expression. Therefore, we selected an MPM cell line resistant to transgene expression, H2052, introduced a CMV promoter-controlled EGFP gene into the cell line using an ADV vector, and searched for methods to increase the reporter gene expression in the cell line.

We previously reported that D-glucose, a less harmful, inexpensive, and readily available saccharide, enhanced transgene expression in mammalian cells [9]. Utilizing the enhancing effect of D-glucose on transgene enhancement, we were able to increase the fluorescence intensity in the EGFP gene-transduced H2052 cells by 1.9 times (Figure 2). However, since even the maximum fluorescence intensity in the D-glucose-treated H2052 cells was only 20.6% of that in H28 cells, it cannot be considered that the transgene expression in H2052 cells was sufficiently upregulated.

Next, we pretreated H2052 cells with an ERK1/2 inhibitor, LY, which has an effect of increasing the transduction efficiency by ADV vectors by increasing the ADV receptors, CAR, on the cell surface. Then, we transduced the cells with an EGFP gene and examined its expression level. However, we found that the fluorescence intensity in the LY-pretreated H2052 cells was increased only 1.7-fold compared to that in the LY-untreated H2052 cells (Figure 3).

Furthermore, we pretreated H2052 cells with an HDAC inhibitor, TSA, that has been reported to increase transgene expression through epigenetic modifications. Following this pretreatment, we introduced an EGFP gene into the cells and then measured its expression. TSA pretreatment at 1 μM increased the fluorescence intensity in H2052 cells to 94.3% of that in the TSA-untreated H28 cells (Figure 4).

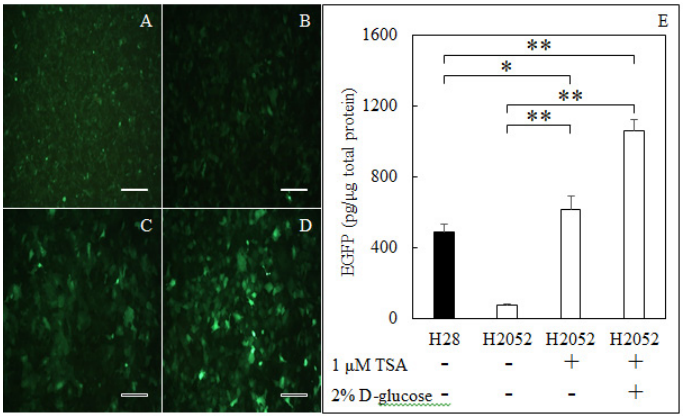

Figure 6. Increase in fluorescence and EGFP amount by the joint effect of TSA and D-glucose in H2052 cells transduced with an EGFP gene using an ADV vector system. We pretreated H2052 cells with 1 μM TSA for 48 h before transduction, introduced a CMV promoter-controlled EGFP gene into H28 and H2052 cells using an ADV vector, and posttreated the transduced H2052 cells by additional 2% D-glucose as described in the Materials and Methods. We then took fluorescence micrographs of the cells **(A-D)** and quantified the amount of EGFP in the cells by ELISA (E) at 48 h after transduction as described in the Materials and Methods. Fluorescence micrographs of H28 cells not treated with TSA or D-glucose **(A)**, H2052 cells not treated with TSA or D-glucose **(B)**, H2052 cells with 1 μM TSA pretreatment and without D-glucose posttreatment **(C)**, and H2052 cells pretreated with 1 μM TSA and posttreated with additional 2% D-glucose **(D)** were taken as described in the Materials and Methods. The internal scale in the fluorescence micrographs represents 200 μm. **(E)** The amount of EGFP was quantified by ELISA and standardized with the amount of total protein. The amount of EGFP was expressed as the amount (pg) per 1 μg total protein. Columns and error bars represent the means of the quadruplicate experiments and their standard deviations, respectively. A black column shows data for H28 cells not treated with TSA or D-glucose and white columns show data for H2052 cells. A single asterisk and double asterisks indicate $p<0.05$ and $p<0.01$, respectively.

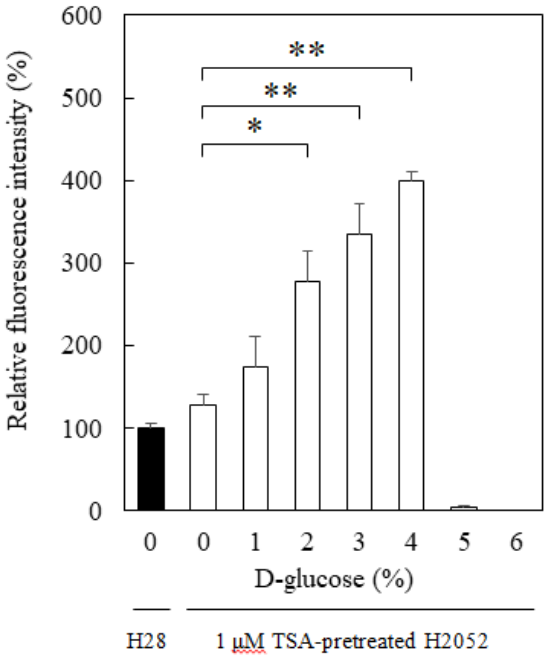

Figure 7. Increase in the amount of PML protein in H2052 cells by TSA treatment. We quantified PML protein levels in H2052 cells treated with 1 μM TSA for 48 h and the TSA-untreated H28 and H2052 cells by ELISA as described in the Materials and Methods. The amount of PML protein was standardized using that of GAPDH measured in the same sample and evaluated relatively. Columns and error bars represent the means of the quadruplicate experiments and their standard deviations, respectively. A black column shows data for H28 cells not treated with TSA and white columns show data for H2052 cells. A single asterisk and a double asterisk indicate $p<0.05$ and $p<0.01$, respectively.

We then pretreated H2052 cells with TSA at 1 μM, transduced the cells with the EGFP gene, and incubated the cells in the presence of D-glucose to determine the joint effect of TSA and D-glucose. The results showed that the fluorescence of cells in the presence of additional 4% D-glucose reached as high as 400% of that in H28 cells not treated with TSA or D-glucose (Figure 5). Fluorescence microscope observation and EGFP quantification also confirmed that this joint effect of TSA and D-glucose ensured sufficient transgene expression in H2052 cells, an MPM cell line resistant to transgene expression (Figure 6).

As to why TSA enhanced the transgene expression in H2052 cells, we can consider two possibilities. One possibility is that TSA inhibited the PML-mediated transcriptional repression, as observed in a previous study [23]. If inhibition of the PML-mediated transcriptional repression by TSA occurred in H2052 cells, the amount of PML protein in the TSA-treated H2052 cells would be expected to be reduced, as well as the results of a related study by Vlasáková J, et al. [22] However, unexpectedly, the PML protein amount in H2052 cells was increased by TSA (Figure 7). This result suggests that the effect of TSA on H2052 cells is not inhibition of the PML-mediated transcriptional suppression, and that the resistance of H2052 cells to transgene expression is not due to the PML-mediated transcriptional suppression.

Another possible explanation for the TSA enhancement of transgene expression in H2052 cells is that TSA induced decondensation of foreign genes in H2052 cells. TSA has been reported to cause extensive chromosome decondensation in cells [15,16]. In their experiments, Bishop CL, et al. hypothesized that many of the foreign genes that they introduced into cells resistant to transgene expression were condensed by the cells in the same way as genes belonging to the heterochromatin region [17]. Transcription from the condensed foreign genes is unlikely to occur because the foreign genes are inaccessible to transcription-associated factors. As hypothesized by Bishop CL, et al. [17] TSA-induced decondensation of foreign genes in H2052 cells may maintain the genes in the lightly packed form found in the euchromatin region and enhance expression of the genes.

When H2052 cells pretreated with 1 μM TSA were cultured in the presence of D-glucose at an additional concentration of 3% or more, extensive cytotoxicity was observed in the cells. It has been reported that TSA may induce apoptosis [24]. It is presumed that the H2052 cells, which had become susceptible to apoptosis by pretreatment with 1 μM TSA, exceeded the threshold for causing apoptosis by adding a D-glucose-induced load.

In this study, the joint effect of TSA and D-glucose was found to greatly elevate transgene expression in MPM cells resistant to transgene expression. Combining this method with recently developed other techniques such as new transcriptional control units to increase transgene expression [25,26] and advanced gene delivery systems [27,28] would be expected to improve the effectiveness of gene therapy.

Conclusion

We identified an MPM-derived cell line in which transgene expression was extremely restricted even after introduction of a foreign gene using a human adenovirus vector, and showed that the combination of TSA and D-glucose greatly elevated transgene expression in this cell line. Combining this method with recently developed other techniques such as new transcriptional control units to increase transgene expression and advanced gene delivery systems would be expected to improve the effectiveness of gene therapy.

Acknowledgement

None.

Conflict of Interest

None.

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