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Suppression of tumor growth by intratumoral injection of short hairpin RNA-expressing plasmid DNA targeting β-catenin or hypoxia-inducible factor 1α

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Abstract

To inhibit the growth of murine melanoma B16 cells in mice, we downregulated the gene expression of β-catenin and hypoxia-inducible factor 1α (HIF1α) in the tumor cells by delivering short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA) targeting one of these genes. Transfection of any of the shRNA-expressing pDNAs to B16 cells resulted in the reduction of the corresponding mRNA, which was associated with a reduced number of viable cells. A flow cytometric analysis of annexin V labeling assay was also performed to count the number of apoptotic cells. A flow cytometric analysis showed that the suppression of the expression of β-catenin or HIF1α in B16 cells increased the number of apoptotic cells. An intratumoral injection of pshβ-catenin (shRNA-expressing pDNA targeting β-catenin) or pshHIF1α (shRNA-expressing pDNA targeting HIF1α) followed by electroporation greatly suppressed the expression of the corresponding target mRNA in the intradermal tumor tissue. The growth of the intradermal tumor was significantly ($P<0.05$) suppressed by the treatment. In conclusion, tumor growth was successfully inhibited by the intratumoral delivery of pshβ-catenin or pshHIF1α.

Keywords: RNAi; Gene delivery; In vivo; Primary tumor; Electroporation

1. Introduction

Tumor cells are characterized by changes in the profile of protein expression. Such changes may lead to the imbalanced production of proteins relating to the malignancy of the cells, or to the production of mutated proteins with abnormal functions. The altered protein expression in tumor cells may contribute to the cell survival, unregulated proliferation and metastatic nature of the cells. Therefore, silencing the expression of these proteins in tumor cells can be a potent and target-specific cancer treatment with a low risk of side effects. RNA interference (RNAi) is a potent and ubiquitous gene silencing mechanism that downregulates the expression of a specific gene of interest [1–5]. After the discovery that short double-stranded RNA (siRNA) can induce RNAi in mammalian cells without stimulating interferon response [6,7], siRNAs have proven to be effective agents for suppressing specific gene expression. The early successes of induction of RNAi in cultured cells have led to high expectations for in vivo and therapeutic applications of siRNAs [8,9]. Cancer is one of the most important target diseases for RNAi-based treatment [10,11]. Contrary to conventional strategies, such as the use of antisense oligonucleotides which have been hampered by low potency and insufficient specificity, the use of siRNAs is reported to be more efficient and to have greater in vivo potency [12–15]. Therefore, RNAi-based gene silencing is a promising approach to achieve target-specific anticancer treatments.

Delivery of siRNA to tumor cells is the greatest obstacle to establishing RNAi as a therapeutic approach to treat cancer because the gene silencing effect is limited to cells that have received siRNA [16–18]. This is a major reason why few papers have reported the successful suppression of in vivo tumor growth by RNAi. In a previous study, we have investigated the

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delivery of siRNA or short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA) to tumor cells in mice [19]. We succeeded in effectively suppressing target gene expression in tumor cells by intratumoral injection of siRNA or shRNA-expressing pDNA followed by electroporation. Using melanoma cells stably expressing firefly luciferase, we found that a single intratumoral injection of shRNA-expressing pDNA following electroporation inhibits the expression by 30%. These results suggest that the delivery of shRNA-expressing pDNA can inhibit the tumor growth if a proper target gene in tumor cells is downregulated by this technique.

In addition to the delivery methods of siRNA, selection of target genes is an important issue that determines the RNAi effects on tumor growth. Although many genes encoding products that play important roles in tumor progression can be targets for RNAi-based suppression of tumor growth, we selected β-catenin and hypoxia-inducible factor 1α (HIF1α) genes as possible target genes to realize RNAi-based cancer therapy. β-catenin plays a key role in cell adhesion and also regulates the activity of certain transcription factors, T cell factor/lymphoid enhancer factor (TCF/LEF) in the Wnt pathway, which activates the transcription of genes related to cell growth and survival [20]. When injected intraperitoneally, siRNA targeting β-catenin complexed with Oligofectamine suppressed the proliferation of colon cancer HCT116 cells in the peritoneal cavity [21]. On the other hand, HIF1 is a heterodimer that consists of constitutively expressed HIF1β and HIF1α, and the expression of the latter is tightly regulated by oxygen concentration. HIF1 activates the transcription of genes that are involved in cancer biology, including angiogenesis, cell survival, glucose metabolism and invasion [22]. Sun et al. reported that intratumoral administration of pDNA which expresses antisense HIF1 suppressed the growth of tumor tissue [23]. In addition, HIF1 was evaluated as a cancer therapeutic target via inducible RNA interference in vivo [24].

Therefore, suppressing the expression of these target genes is expected to inhibit tumor growth. To suppress the expression of these target genes, we constructed and used shRNA-expressing pDNA, not siRNA, to induce RNAi in tumor cells, because (i) shRNA-expressing pDNA shows more sustained RNAi effects than siRNA, and (ii) there was little difference in the delivery efficiency to tumor cells between shRNA-expressing pDNA and siRNA. We report here that shRNA-expressing pDNA targeting β-catenin or HIF1α effectively suppresses tumor growth in mice following intratumoral injection followed by electroporation.

2. Materials and methods

2.1. shRNA-expressing pDNA

shRNA-expressing pDNAs driven by human U6 promoter were constructed from piGENE-hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to the manufacturer's instructions. Target sites in murine genes encoding β-catenin and HIF1α were as follows: β-catenin site1, 5′-GCGGTAGGG TAAATCAGTA-3′, site2, 5′-GAATGAGACTGCAGATCTT-3′; HIF1α site1, 5′-GTGAAAGGATTCATATCTA-3′, site2, 5′-GACACAGCCTCGATATGAA-3′. These pDNAs transcribe a stem-loop-type RNA with loop sequences of ACG UGU GCU GUC CGU. piGENE-hU6 vector, which transcribes a non-related sequence of RNA with partial duplex formation, was used as a control pDNA throughout the present study. Each pDNA was amplified in the DH5α strain of *Escherichia coli* and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

2.2. Cell culture

A murine melanoma cell line B16-F1, B16-BL6 and B16-BL6 cells that stably express firefly luciferase and sea pansy luciferase (B16-BL6/dual Luc) were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/L-glutamine (PSG) at 37 °C and 5% CO_2/95% air [19].

2.3. In vitro transfection

B16 cells were plated on culture plates. After an overnight incubation in DMEM containing 10% FBS and PSG at 37 °C in 5% CO_2/95% air, transfection of pDNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, 1 μg pDNA was mixed with 3 μg Lipofectamine 2000 at a final concentration of 2 μg pDNA/ml dissolved in OPTI-MEM I (Invitrogen), and the resulting complex was added to the cells and the cells were incubated with the complex for 4 h. Cells were washed with PBS and further incubated with the culture medium as described above for specified time periods up to 96 h.

2.4. mRNA quantification

Total RNA was isolated using MagExtractor MFX-2100 and a MagExtractor RNA kit (TOYOBO, Osaka, Japan) following the manufacturer's protocol. To eliminate DNA contamination, the total RNA was treated with DNase I (Takara Bio, Otsu, Japan) prior to reverse transcription. Reverse transcription was performed using a SuperScript II (Invitrogen) and dT-primer following the manufacturer's protocol. For quantitative mRNA expression analysis, real-time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basle, Switzerland). The sequences of the primers used for amplification were as follows: GAPDH forward, 5′-CTGCCA AGTATGATGACATCAAGAA-3′, reverse, 5′-ACCAGGAAA TGAGCTTGACA-3′; β-catenin forward, 5′-CCTGCAGAAC TCCAGAAAG-3′, reverse, 5′-GTGGCAAAAACATCAAC GTG-3′; HIF1α forward, 5′-TCAAGTCAGCAACGTGGA AG-3′, reverse, 5′-TATCGAGGCTGTGTCGACTG-3′. Amplified products were detected on-line via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics). The cycling conditions were as follows: initial enzyme activation at 95 °C for 10 min, followed by 55 cycles at 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 20 s. Gene-specific fluorescence was measured at

72 °C. The mRNA expression of target genes was normalized by using the mRNA level of GAPDH.

2.5. Effect of shRNA-expressing pDNAs on proliferation of B16 cells

B16-BL6/dual Luc cells were transfected with pDNA as described above. After the transfection of pDNAs for 4 h, cells were washed with PBS and further incubated with the culture medium for specified time periods up to 96 h. The cell numbers at the indicated time points were evaluated by MTT assay as described previously [25].

2.6. Flow cytometric determination of apoptosis

B16-BL6/dual Luc cells seeded on 6-well culture plates were treated with pDNA as described above. At 3 days after transfection, adherent cells were detached by trypsinization and re-suspended in PBS. Aliquots of these cell suspensions were centrifuged and the pellets were used for the flow cytometric determination of apoptosis using a commercial kit (Vybrant™ Apoptosis Assay Kit #2, Molecular Probes, Invitrogen). In brief, a cell pellet was re-suspended in a binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) to give a cell density of approximately $2–10\times10^5$/ml. Aliquots (100 μl) of this cell suspension were incubated with 5 μl recombinant annexin V labeled with Alexa Fluor® 488 dye (Alexa Fluor 488 annexin V) and 1 μl of a stock solution of propidium iodide (PI, 100 μg/ml) for 15 min at room temperature. After the incubation period, 400 μl of the binding solution was added and the samples were kept on ice until analysis in the flow cytometer. Samples were analyzed on a flow cytometer (FACSCan, BD, Franklin Lakes, NJ, USA) and electronic compensation was used to remove spectraloverlap. Annexin V staining, DNA signals and side scatter (SSC) signals were detected on a log scale. Forward (FSC) signals were detected in a linear mode. The FL1 and FL2 photomultiplier (PMT) voltage settings were set using unstained isotype samples. The threshold using FSC was set to exclude debris without excluding any populations of interest. The flow cytometric data were analyzed with WINMDI 2.8 software®.

2.7. Animals

Seven-week-old male C57/BL6 mice, purchased from Shizuoka Agricultural Cooperative Association (Shizuoka, Japan) were used in all the experiments. All animal experiments were conducted in accordance with the principles and procedures outlined in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University.

2.8. Primary tumor model

B16-BL6 cells in an exponential growth phase were harvested by trypsinization and suspended in Hanks' balanced salt solution (HBSS, Nissui Pharmaceutical). The tumor cells (2×10^5 cells) were injected intradermally into the back of syngeneic C57/BL6 mice. When the tumor diameter reached 2–3 mm (1 week after tumor inoculation), mice received a single intratumoral injection of 30 μg pDNA dissolved in 50 μl saline at a time followed by twelve electric pulses (1000 V/cm, 5 ms, 4 Hz) which were delivered through a pair of 1-cm^2 forcep-type electrodes connected to a rectangular direct current generator (CUY21, Nepagene, Chiba, Japan). In the growth inhibition experiment, shRNA-expressing pDNAs were administrated at day 7, 10 and 19 after tumor inoculation. Tumor growth was evaluated by measuring the tumor size using calipers at indicated times, and tumor volume (mm^3) was calculated from the following equation: $(\text{longest diameters}\times\text{shortest diameters})^{3/2}\times\pi/6$.

2.9. Statistical analysis

Differences were statistically evaluated by Student's *t*-test. A *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Reduction in mRNA by transfection of shRNA-expressing pDNA to B16-F1 cells

Two candidate sequences were selected for each target gene. Fig. 1 shows the mRNA levels of the target genes in B16-F1 cells 24 h after transfection of shRNA-expressing pDNA targeting β-catenin or HIF1α measured by real-time RT-PCR. The transfection of shRNA-expressing pDNA maximally suppressed the mRNA expression of β-catenin and HIF1α to about 20% and 25% of each control value, respectively. For each target gene, an shRNA-expressing pDNA (#2 in all cases)

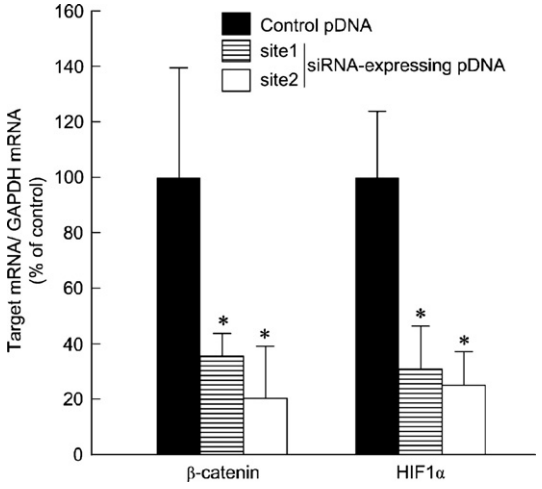

Fig. 1. Reduction of mRNA in B16 cells following transfection of shRNA-expressing pDNA. B16-F1 cells seeded on 12-well culture plates (at a density of 2×10^5 cells/well) were transfected with shRNA-expressing pDNA or piGENE-hU6. Amounts of mRNA were determined 24 h after transfection. The results are expressed as the mean±S.D. ($n=3$). *$P<0.05$ for Student's *t*-test versus control group.

was selected based on the inhibitory effect, and was used in the subsequent experiments. The selected shRNA-expressing pDNAs were named as pshβ-catenin and pshHIF1α.

3.2. Growth inhibition of B16-BL6/dual Luc cells by shRNA-expressing pDNA

To evaluate the inhibitory effect of shRNA-expressing pDNA on tumor cell growth in vitro, the time course of the number of B16-BL6/dual Luc cells after the transfection of shRNA-expressing pDNAs was measured by MTT assay (Fig. 2). Transfection of pshβ-catenin or pshHIF1α reduced the number of viable B16 cells to about 50% and 60% of the control value, respectively. A significant reduction in the viable cell number was observed in the pshβ-catenin- or pshHIF1α-treated cells as early as 1 and 2 days after transfection, respectively. Therefore, these results indicate that pshβ-catenin and pshHIF1α are potent in inhibiting the proliferation of B16 cells.

3.3. Flow cytometric analysis of cell death induced by shRNA-expressing pDNAs

Flow cytometric analysis of cells stained with fluorescein-labeled annexin V and PI was performed to detect apoptotic and necrotic cells after transfection of shRNA-expressing pDNAs to B16-BL6/dual Luc cells (Fig. 3). At 3 days after transfection of shRNA-expressing pDNA, cells were stained with annexin-FITC and PI. Fig. 3A–C shows the typical results of the flow cytometric analysis of the cells. Live cells (annexin-FITC and PI double negative) occupy the lower left quadrant, early apoptotic cells (annexin-FITC positive and PI negative) occupy the lower right quadrant and late apoptotic or necrotic cells (annexin-FITC and PI double positive) occupy the upper right quadrant. Fig. 3D summarizes the number of viable, early apoptotic, or late apoptotic and necrotic cells. Transfection of pshβ-catenin or pshHIF1α increased the number of apoptotic cells compared with that of control pDNA. The number of dead cells, which

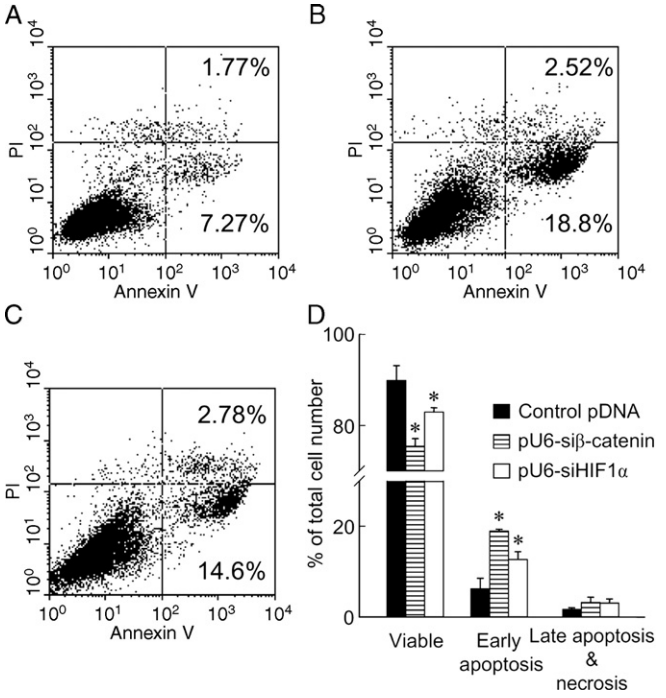

Fig. 3. Induction of apoptosis and necrosis in B16-BL6 cells caused by transfection of shRNA-expressing pDNA. (A–C) Representative dot plots of the flow cytometric quantification of intact, apoptotic and necrotic cells. B16-BL6/dual Luc seeded on 6-well culture plates (at a density of 1×10^5 cells/well) were transfected with piGENE-hU6 (A), pshβ-catenin (B) or pshHIF1α (C). After 3 days incubation from the initiation of transfection, cells were processed and stained with annexin-FITC and PI as indicated in M&M. Live cells (annexin-FITC and PI double negative) occupy the lower left quadrant, early apoptotic cells (annexin-FITC positive and PI negative) occupy the lower right quadrant and late apoptotic or necrotic cells (annexin-FITC and PI double positive) occupy the upper right quadrant. (D) Percentage of viable, early apoptotic and late apoptotic or necrotic cells after transfection of shRNA-expressing pDNA, as calculated from the dot plots as in panel (A–C). The results are expressed as the mean±S.D. ($n=3$). *$P<0.05$ for Student's t-test versus control group.

was roughly estimated as the total number of apoptotic and necrotic cells, was about 20% of the total cells in the case of pshβ-catenin, 15% in the case of pshHIF1α and 10% in the case of control pDNA.

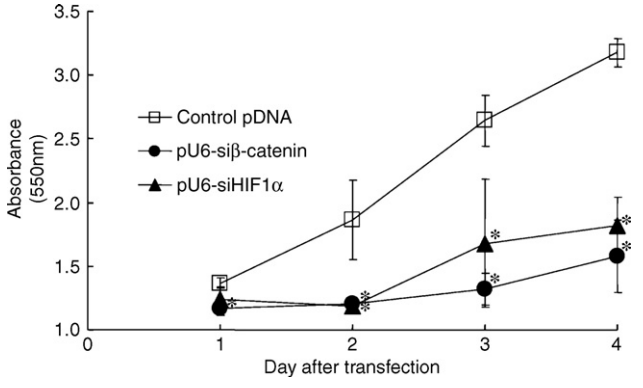

Fig. 2. Anti-proliferative effect of shRNA-expressing pDNA against B16 cells. B16-BL6/dual Luc seeded on 24-well culture plates (at a density of 2×10^4 cells/well) were transfected with shRNA-expressing pDNA or piGENE-hU6. Cell populations at indicated time points were evaluated by MTT Assay. The results are expressed as the mean±S.D. ($n=3$). *$P<0.05$ for Student's t-test versus control group.

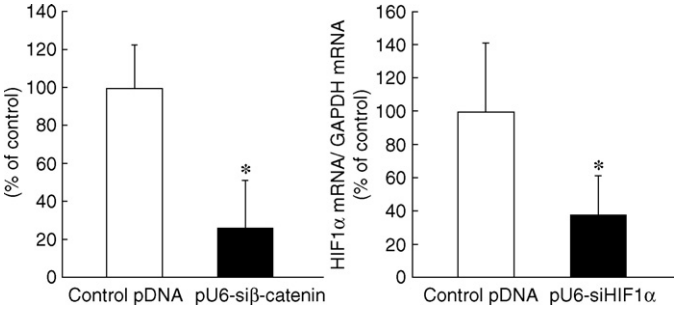

Fig. 4. Reduction of mRNA expression in primary tumor tissue by intratumoral injection of shRNA-expressing pDNA followed by electroporation. Mice received an intratumoral injection of piGENE-hU6 or shRNA-expressing pDNA (30 μg), followed by electroporation. Amounts of mRNA in tumor tissue were determined 24 h after administration. The results are expressed as the mean±S.D. ($n=4$). *$P<0.05$ for Student's t-test versus control group.

3.4. Reduction of mRNA and growth inhibition of subcutaneous tumor tissue of B16-BL6/dual Luc by intratumoral injection of pshβ-catenin or pshHIF1α

Fig. 4 shows the amount of β-catenin and HIF1α mRNA in B16 tumor tissue in mice 24 h after intratumoral injection of pshβ-catenin or pshHIF1α followed by electroporation. A single injection of each shRNA-expressing pDNA reduced the amount of the corresponding target mRNA in the tumor tissue. The mRNA level of β-catenin and HIF1α was reduced to 25 and 35% of the control value, respectively, demonstrating that intratumoral injection of shRNA-expressing pDNA can efficiently suppress the corresponding target gene expression in tumor tissue in mice.

To suppress the tumor growth in mice, tumor-bearing mice received intratumoral injection of shRNA-expressing pDNA followed by electroporation at day 7, 10 and 19 days after tumor inoculation. The injection of control pDNA followed by electroporation retarded the growth of tumor tissue compared with the no treatment group (data not shown). Intratumoral delivery of pshβ-catenin or pshHIF1α significantly reduced the growth of the tumor tissue compared with the delivery of control pDNA (Fig. 5A); the tumor volume was significantly suppressed by intratumoral delivery of pshβ-catenin or pshHIF1α 18 or 15 days after initiation of the therapeutic treatment, respectively. Tumor growth was suppressed in all mice treated with pshβ-catenin or pshHIF1α, and the tumor regressed markedly in two and one out of the four mice treated with pshβ-catenin or pshHIF1α, respectively, as shown in Fig. 5B–D.

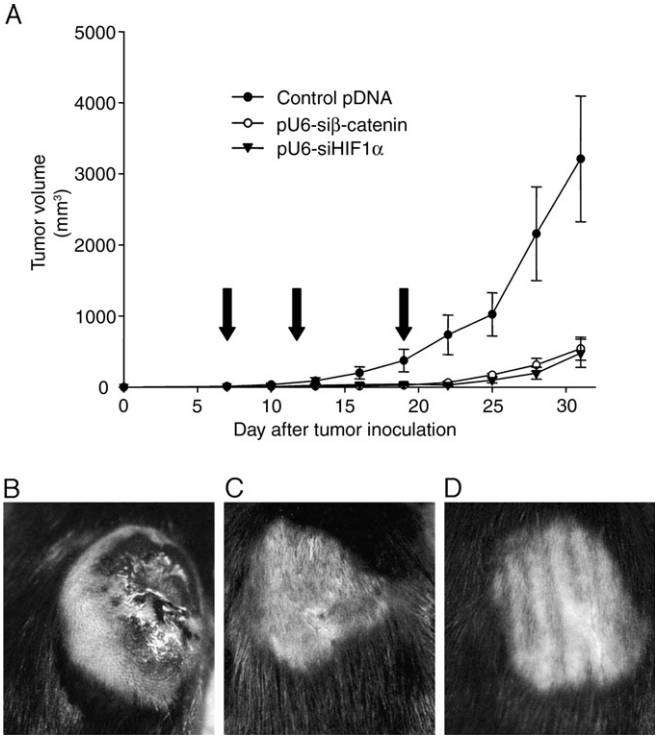

Fig. 5. Effects of intratumoral delivery of shRNA-expressing pDNAs on the growth of primary tumor tissue. (A) Mice received an intratumoral injection of 30 μg piGENE-hU6 or shRNA-expressing pDNAs followed by electroporation. shRNA-expressing pDNAs were administrated at day 7, 10 and 19 after tumor inoculation. Arrows indicate the timing of pDNA administration. The results are expressed as the mean±S.E.M. (n=4). *P<0.05 for Student's t-test versus control group. (B–D) Photographic image of tumor tissue of mice who received an intratumoral injection of piGENE-hU6 (B), pshβ-catenin (C) or pshHIF1α (D) at 18 days after initiation of therapeutic treatment.

4. Discussion

When B16 cells were transfected with pDNA expressing enhanced green fluorescent protein (EGFP) in vitro, a flow cytometric analysis demonstrated that about 80% of cells were EGFP positive at 24 h after transfection (unpublished data). Therefore, pDNA expressing shRNA can also be delivered to about 80% of cells in the transfection condition used, because the two plasmids are not quite different in size (3 kb for pDNA expressing shRNA and 4 kb for pDNA expressing EGFP). Transfection of pshβ-catenin and pshHIF1α was effective in suppressing corresponding target mRNA expression to about 20 or 25% of control value, respectively. Reduction in the mRNA expression of β-catenin or HIF1α was associated with a reduced number of viable cells. There are two different mechanisms governing the reduction in the proliferation of B16 tumor cells by the suppression of β-catenin or HIF1α expression: one is a reduced proliferation rate of B16 cells [21,22], and the other is an increased number of dead cells. To investigate whether the transfection of pshβ-catenin or pshHIF1α increases the number of dead cells, B16 cells were double-stained with annexin V conjugated with fluorescein and PI, and analyzed by flow cytometry to determine the ratio of dead cells to total cells. To analyze the growth and death rate of the transfected B16 cells, we assumed that cells proliferate and die according to first-order rate processes. The death rates of cells were calculated based on the data shown in Fig. 3D. To calculate the proliferation rates, the time course of the viable cell number after transfection was evaluated by simultaneously performing an MTT assay (data not shown). The calculated proliferation rates were 0.485 (control pDNA), 0.270 (pshβ-catenin) and 0.412 (pshiHIF1α), and the death rates of the cells were 0.0286 (control pDNA), 0.0948 (pshβ-catenin) and 0.0621 (pshHIF1α). These results suggest that both the increase in cell death and the decrease in cell proliferation rate contribute to the decrease in the number of B16 cells caused by the transfection of pshβ-catenin and pshHIF1α.

We have reported that electroporation significantly (about 10-fold) increases transgene expression in tumor tissues after intratumoral injection of pDNA expressing firefly luciferase [19]. Electroporation was also effective in suppressing target gene expression after intratumoral injection of shRNA-expressing pDNA. Intratumoral injection of shRNA-expressing pDNA suppressed target gene (firefly luciferase) expression to about 30% of the control value when combined with electroporation [19], but to about 80% without electroporation (unpublished data). Therefore, the delivery efficiency of shRNA-expressing pDNA can also be significantly increased by electroporation. An intratumoral injection of pshβ-catenin or pshHIF1α followed by electroporation successfully suppressed the corresponding target gene expression to the level below

30% of the control. Moreover, intratumoral delivery of pshβ-catenin or pshHIF1α also retarded in vivo tumor growth although the mRNA expression was not completely suppressed. Intratumoral injection of shRNA-expressing pDNAs followed by electroporation reduced target gene expression to about 30% of the control values, and the intensity of inhibitory effect was comparable with that of in vitro transfection, in which about 80% of cells receive plasmid DNA as mentioned above. Therefore, these results suggest that about 80% of the tumor cells receive the shRNA-expressing pDNA injected intratumorally, and that the delivery efficiency is enough to suppress tumor growth. Large tumor tissues with a diameter of over 6 mm were less sensitive to the same treatment than small ones of 2–3 mm in diameter (data not shown), suggesting the importance of the delivery efficiency of the shRNA-expressing pDNA. A combination with other therapeutic treatments, such as enhancing host immune response to tumor cells, could be an effective approach to further suppressing tumor growth.

5. Conclusion

We found that the silencing of β-catenin or HIF1α is effective in reducing the proliferation of melanoma cells. The delivery of pshβ-catenin or pshHIF1α to intradermal tumor tissues suppressed the corresponding mRNA expression and the growth of tumor tissue. These results indicate that strategies targeting β-catenin or HIF1α, including the use of RNAi, may be of use in the future treatment of cancer.

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