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Vesicular Stomatitis Virus G Glycoprotein and ATRA Enhanced Bystander Killing of Chemoresistant Leukemic Cells by Herpes Simplex Virus Thymidine Kinase/Ganciclovir

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Abstract

Refractoriness of acute myeloid leukemia (AML) cells to chemotherapeutics represents a major clinical barrier. Suicide gene therapy for cancer has been attractive but with limited clinical efficacy. In this study, we investigated the potential application of herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) based system to inhibit chemoresistant AML cells. We first generated Ara-C resistant K562 cells and doxorubicin-resistant THP-1 cells. We found that the HSV-TK/GCV anticancer system suppressed drug resistant leukemic cells in culture. Chemoresistant AML cell lines displayed similar sensitivity to HSV-TK/GCV. Moreover, HSV-TK/GCV killing of leukemic cells was augmented to a mild but significant extent by all-trans retinoic acid (ATRA) with concomitant upregulation of Connexin 43, a major component of gap junctions. Interestingly, HSV-TK/GCV killing was enhanced by expression of vesicular stomatitis virus G glycoprotein (VSV-G), a fusogenic membrane protein, which also increased leukemic cell fusion. Co-culture resistant cells expressing HSV-TK and cells stably transduced with VSV-G showed that expression of VSV-G could promote the bystander killing effect of HSV-TK/GCV. Furthermore, combination of HSV-TK/GCV with VSV-G plus ATRA produced more pronounced antileukemia effect. These results suggest that the HSV-TK/GCV system in combination with fusogenic membrane proteins and/or ATRA could provide a strategy to mitigate the chemoresistance of AML.

Key Words: VSV-G, ATRA, Bystander killing, Chemoresisitant leukemia cells, HSV-TK/GCV

INTRODUCTION

Despite the progresses made in acute myeloid leukemia (AML) therapy, the outcomes of most AML patients remain poor. The 5-year survival of patients with AML was reported to be around 20% (Pulte *et al*., 2010). Resistance of AML cells to chemotherapy is believed to be a major cause for the treatment failures. However, the biological mechanisms underlying the chemoresistance of AML are unclear. Our earlier work demonstrated that Nf1 deficiency and the loss-of-function mutation in p53 contributed to resistance of AML cells to Ara-C, indicating these genetic changes are responsible for chemotherapeutic responses of AML (Yin *et al*., 2006a; Yin *et al*., 2006b). Accordingly, a MEK inhibitor and a p53 regulator showed suppressive effects on resistant AML cells (Yin *et al*., 2006a; Yin *et al*., 2006b). Although strategies have been developed to overcome the chemoresistance of AML, their clinical efficacies have not been demonstrated yet. It is necessary to develop new approaches for the treatment of chemoresistant AML.

Gene therapy strategies for leukemia have been pursued for nearly two decades (Braun *et al*., 1997). Suicide gene therapy is one of several gene therapeutic approaches to treat cancer, often utilizing a gene encoding a protein which can convert a nontoxic prodrug into toxic metabolites that kill the genetically modified cell (Dilber and Gahrton, 2001). A variety of suicide systems have been characterized, including HSV-TK/GCV, UPRT/5-FU, cytosine deaminase from bacteria or yeast with 5-fluorocytosine, and bacterial nitroreductase with 5-(azaridin-1-yl)-2,4-dinitrobenzamide, and their respective derivatives (Kawamura *et al*., 2001; Dachs *et al*., 2009; Du-

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arte *et al*., 2012). Among them, the HSV-TK/GCV system receives much attention and its therapeutic activity has been broadly examined in many forms of cancers. Upon GCV treatment, GCV gets into cells and converted into an active form following phosphorylation by thymidine kinase encoded by HSK-TK. This active triphosphorylated GCV can then incorporate into DNA being synthesized and thereby stop DNA replication which subsequently leads to a killing effect on target cells. HSV-TK/GCV suicide gene approach has demonstrated therapeutic effects against hematologic malignancies (Blumenthal *et al*., 2007; Miyake *et al*., 2007). HSV-TK/GCV has also been used to control severe graft-versus-host disease in mouse models and clinical trials of allogeneic hematopoietic stem cell transplantation (Onodera, 2008; Bondanza *et al*., 2011; Borchers *et al*., 2011; Casucci *et al*., 2013). Because the low efficiency in gene delivery remains a major challenge to gene therapy, however, the clinical success of suicide gene approach for cancer therapy is limited, which highlights the need for the development of more robust approaches (Neschadim *et al*., 2012).

Some suicide gene products induce a so-called 'bystander effect', typical of HSV-TK/GCV system (Dilber and Gahrton, 2001; Van Dillen *et al*., 2002). The bystander effect is a toxic effect of targeted cells on adjacent nontargeted cells and sometimes on distant cells as well, probably due to transfer of toxic metabolites or signals. Because suicide gene cannot be easily introduced into the whole cell population of a tumor, the successful eradication of tumors depends on the bystander effect (Mesnil and Yamasaki, 2000). Therefore, boosting the bystander effect may represent a strategy to increase the therapeutic activity of HSV-TK/GCV. The bystander effect is most evident in tumour cells that have a high number of gap junctions build up by connexins and thus, may be modulated by manipulation of gap junctions (Dilber and Gahrton, 2001). VSV-G is a pH-dependent fusogenic membrane glycoproteins (FMGs), convenient to use without the need for activation by additional drugs or special enzymes, and relatively less biohazardous. Cells expressing FMG can form syncytia with adjacent cells (Hoffmann *et al*., 2007). ATRA potently induces the differentiation of leukemic cells, and is clinically used as a first frontline drug to treat APL (Yang *et al*., 2012). Whether these approaches and agents can aid the bystander effect of HSV-TK/GCV requires determination.

In this study, we investigated whether the most commonly employed suicide gene therapy system, HSV-TK/GCV, can be used to treat chemoresistant AML cells. We established leukemic cells resistant to Ara-C and doxorubicin, respectively. We found that the HSV-TK/GCV system could effectively suppress drug resistant leukemic cells in culture, and different leukemia cell lines had similar suscepibility to HSV-TK/GCV killing. The HSV-TK/GCV suppressive effect could be enhanced by VSV-G or ATRA. This enhancement was demonstrated to act through an increased bystander effect of HSV-TK/GCV. Combination of HSV-TK/GCV with VSV-G plus ATRA resulted in even more pronounced antileukemia effect. This work may aid the treatment of chemoresistant AML using suicide gene therapy.

MATERIALS AND METHODS

Cell culture, drugs and chemicals

Human leukemic cell lines K562 and THP-1 and their derivatives were grown in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin, in a humidified atmosphere containing 5% CO_2 at 37°C. All culture media and supplements, except noted individually, were obtained from Invitrogen (Carlsbad, CA, USA). Ara-C was purchased from Pfizer Italia S.R.L (Neriviano, Italy). Doxorubicin was obtained from Main Luck (Shenzhen, China). Other drugs and reagents used in this study included GCV (Ke Yi, Hubei, China) and ATRA (Sigma, St. Louis, MO, USA). For all the studies described below, cells growing in logarithmic phase of growth were used.

Construction of retroviral vector and transduction of AML cell lines

The VSV-G and HSV-TK cDNAs were inserted into the retroviral expression vectors pMIGR-his and pMSCV-puro (expressing GFP and puromycin-resistant proteins, respectively), to generate the pMIGR-VSV-G-his and pMSCV-TK-puro constructs, respectively. These constructs or the empty vectors were co-transfected with plasmids encoding packaging protein VSV-G and MuLV gag-pol, by calcium phosphate precipitation into 293T cells, to produce retroviruses, as described before (Yin *et al*., 2009). Retroviral supernatants were exposed to leukemic cells in the presence of 4 mg/mL polybrene. The stably transduced cells were either sorted out by fluorescence-activated cell sorting using EGFP, or selected using puromycin-resistant proteins, expressed by these constructs, respectively.

RT-PCR and quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using TRIzol (Molecular Research Center, Cincinnati, OH, USA) approach, as described previously (Yin *et al*., 2006a). 0.1 ug of total RNA was reverse transcribed with oligo (dT)n, using the SuperScript II First Strand cDNA Synthesis system (Invitrogen) according to the manual. The specific primers in PCR for detecting expression of HSV-TK were 5'-TTATGGCTTCGTACCCCGGCCA-3' (forward) and 5'-TTGTTCTGTCTTTTTATTG-3' (reverse), and of VSV-G were 5'-TTATGAAGTGCCTTTTGTACTT-3' (forward) and 5'-TTGCTTTCCAAGTCGGTTCATC-3' (reverse), repectively. QPCR was performed using SYBR Green method (Applied Biosystems, Foster City, CA, USA). Individual reaction contains diluted cDNA, 400 nM forward and reverse primers each, and 12.5 μl 2×PCR master mixes. PCR reaction was denatured at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using ABI Prism 7500 SDS Software. The specific primers used for amplification of Connexin 43 were 5'-CGCCTATGTCTCCTCCTG-3' (forward) and 5'-ACAATTACCACCGCTCA-3' (reverse), and GAPDH transcript levels for normalization 5'-GCCAAGGTCATCCATGACAACT-3' (forward) and 5'-GCCATCACGCCACAGTTTC-3' (reverse), respectively.

Western blotting

Expression of VSV-G protein was detected by Western blotting with anti-His tag antibody, as previously described (Yin *et al*., 2006a). Briefly, cells were lysed in WB cell lysis buf-

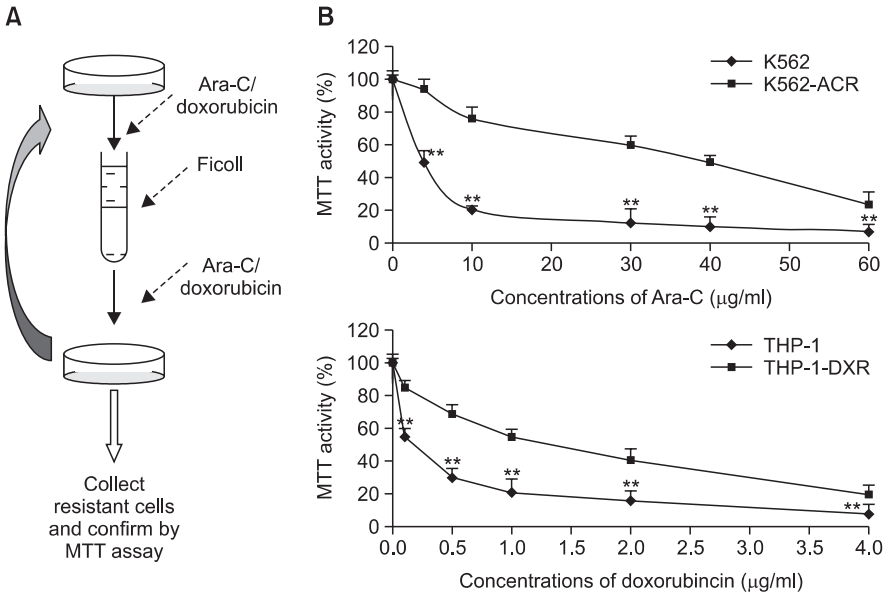

Fig. 1. Generation of chemoresistant leukemic cells. (A). Selection scheme for derivation of leukemic cells resistant to Ara-C (K562-ACR) or doxorubicin (THP1-DXR). (B) Measurement of the sensitivities of K562-ACR and THP1-DXR cells to Ara-C and doxorubicin, respectively. Cells were exposed to chemotherapeutics for 3 days prior to MTT assay. ***p*<0.01. Data shown here are representative of three experiments.

fer (Beyotime, Shanghai, China) supplemented with protease inhibitor cocktail (Roche, Palo Alto, CA, USA) to obtain proteins. Protein concentrations were determined by the Bradford method (Pierce, Rockford, IL, USA). Thirty micrograms of total proteins were boiled and subsequently resolved by electrophoresis on 12% SDS-PAGE gels, and blotted to methanol-activated PVDF membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked and probed with the primary anti-His tag (1:3000, Santa Cruz) and rabbit polyclonal anti-β-actin (1:2000, Sigma) antibodies, followed by incubation with the secondary antibodies Goat polyclonal anti-rabbit (both at 1:1000, LI-COR,USA).

Cytotoxicity assays

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Amresco, USA) tetrazolium assay was used to determine cytotoxic response of AML cells, as described before (Yin *et al*., 2006a). 5000~7000 cells/well were plated into flat-bottom 96-well plates (Corning Inc, Corning, NY, USA) in 200 μl of culture medium containing various concentrations of GCV as indicated for three days. 20 μl of MTS solution was added to each well and incubated at 37°C for 2 hours. The optical density at 650 nm was recorded as a reference, and subtracted from OD490 readings to eliminate nonspecific absorbance. Data from individual experiments are presented as the mean percentage of corrected OD490 of triplicate cultures ± SD.

Flow cytometry analysis of cell apoptosis

Leukemic cells were stained with Propidium iodide (BD Pharmingen, NJ, USA) and Annexin V-FITC (Biouniquer, Nanjing, China) following the manufacturer's protocol. After staining, cells were washed and analyzed on a FACScan (BD Biosciences, Franklin Lakes, USA) using FlowJo software (Tree-Star Inc, Ashland, USA).

Statistical analysis

Data of cell survival and apoptosis were expressed as mean ± SD. Two-tailed Student's *t*-tests were performed using Microsoft Excel. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Establishment of leukemic cells resistant to Ara-C or doxorubicin

To generate Ara-C resistant leukemic cells, we treated K562 and THP-1 cells with 3 μg/mL of Ara-C as the initial selection. After 2-3 days of incubation, dead cells and debris were removed using Ficoll separation. Surviving cells were subsequently exposed to increasing concentrations of Ara-C. Our selection scheme is depicted in Fig. 1A. Following this procedure, Ara-C resistant K562 cells (K562-ACR) were obtained after 10 weeks of selection. However, THP-1 cells failed the Ara-C selection. Likewise, THP-1 cells resistant to doxorubicin (THP1-DXR) were derived following 11 weeks of selection. The sensitivity of K562-ACR cells to Ara-C and that of THP1-DXR cells to doxorubicin were determined using MTS assay to be 9.9 fold and 8.12 fold those of their parental cells, respectively (Fig. 1B).

HSV-TK/GCV system suppressed chemoresistant leukemic cells

In order to determine the effect of HSV-TK/GCV on resistance cells, we generated HSV-TK stably transduced K562-ACR (K562-ACR-TK) and THP1-DXR (THP1-DXR-TK) cells through selection for puromycin resistance. The K562-ACR-TK and THP1-DXR-TK cells expressed HSV-TK RNA, as detected by RT-PCR (Fig. 2B). A three-day exposure of these cells to GCV at the indicated concentrations resulted in a sig-

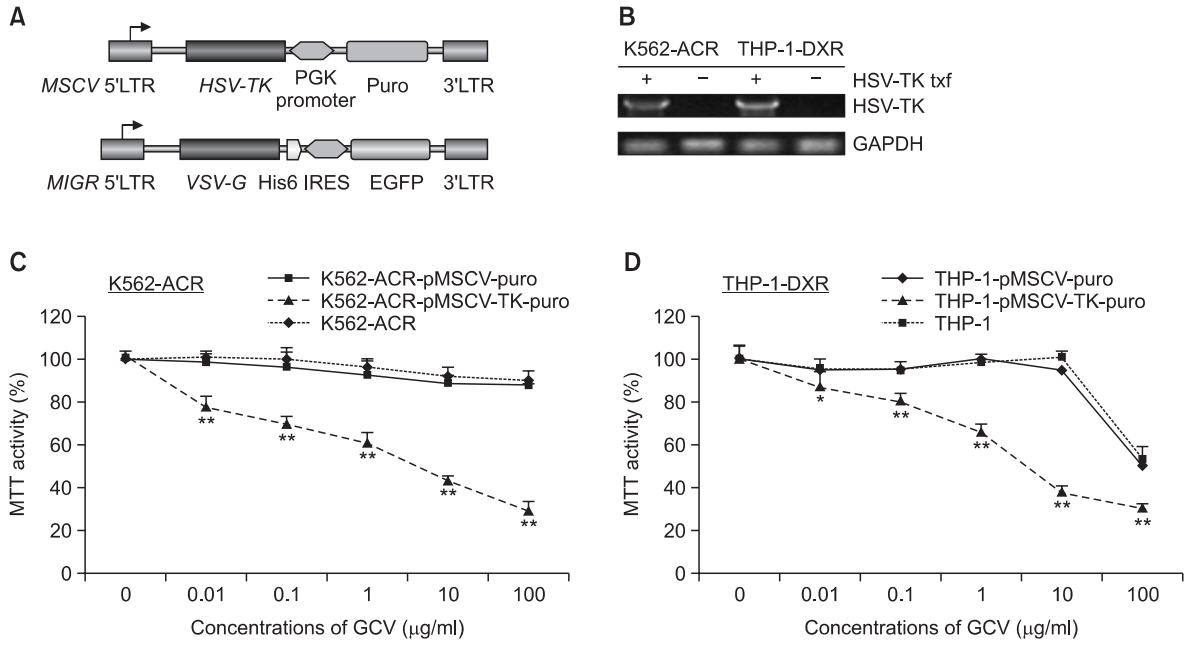

Fig. 2. Effect of HSV-TK/GCV system on chemoresistant leukemic cells. (A) Schematic representation of the constructs used in this study. (B) Expression of HSV-TK in chemoresistant K562-ACR and THP1-DXR cells as determined by RT-PCR. Shown here are the electrophoretic results of RT-PCR products amplified for HSV-TK gene, with GAPDH as the internal control. txf: transfection. (C) Analysis of cell survival following a 3-day exposure to an escalating concentrations of GCV, as measured by MTT assay (detailed in the Methods). *p<0.05; **p<0.01. Data shown here are representative of three experiments.

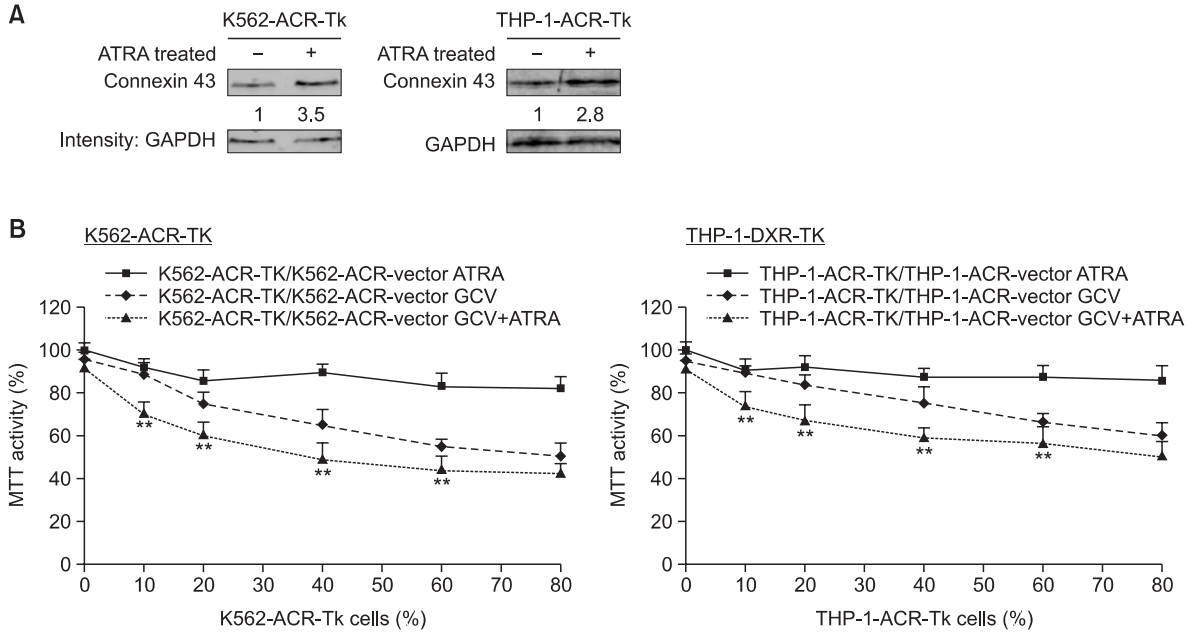

Fig. 3. Expression of Connexin 43 and augmented bystander effect of HSV-TK/GCV following ATRA treatment. (A) ATRA treatment upregulated expression of Connexin 43 in chemoresistant leukemic cells. K562-ACR-TK and THP1-DXR-TK cells were treated with 0.1 μM ATRA for 72 hours and subsequently subjected to western blotting assay. The relative strength of signal for connexin 43 was quantified using program Image J, as shown below the connexin 43 panel. (B) ATRA enhanced the bystander effect of HSV-TK/GCV on chemoresistant cells. HSV-TK expressing cells and non-expressing cells (left: K562-ACR-TK; right: THP1-DXR-TK) were co-cultured at the ratio ranging from 0% to 60%, and exposed to 100 μg/mL (for K562) or 10 μg/mL (for THP-1) GCV in the absence or presence of 0.1 μM ATRA for 3 days, followed by MTT assay. *p<0.05; **p<0.01. Data shown here are representative of at least three experiments.

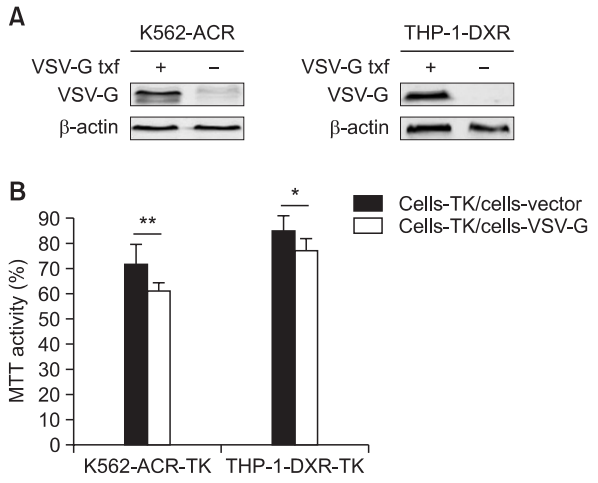

Fig. 4. Effect of VSV-G expression on the killing by HSV-TK/GCV. (A) Expression of VSV-G in K562-ACR (left) and THP-1-DXR (right) cells, as detected by western blotting assay. txf: transfection. (B) Effect of VSV-G expression on the bystander effect of HSV-TK/GCV. HSV-TK expressing cells and VSV-G-expressing cells were co-cultured at the ratio of 1:4 in the presence of 100 μg/mL (for K562) or 10 μg/mL (for THP-1) GCV for 3 days, followed by MTT assay. Co-culture of HSV-TK expressing cells with empty vector-transduced cells at the ratio of 1:4 was set as control. **p*<0.05; ***p*<0.01. Data shown here are representative of at least three experiments.

nificant decrease ($p<0.05$) in cell survival, compared to non-tranduced or empty vector-tranduced cells (Fig. 2C, D). This indicated that chemoresistant leukemic cells were susceptible to HSV-TK/GCV killing. In addition, K562-ACR-TK and THP1-DXR-TK cells showed similar IC50 of GCV (Fig. 2C, D), indicating that HSV-TK/GCV system suppressed both chemoresistant leukemic cell lines with a comparable efficiency.

ATRA treatment upregulated connexin43 expression and augmented HSV-TK/GCV efficacy

Since HSV-TK/GCV has limited clinical success, and ATRA was shown by other studies to promote bystander effect, we attempted to determine the effect of ATRA on HSV-TK/GCV killing of drug resistant cells. As can be seen in Fig. 3A, treatment with ATRA at the concentration of 1×10^{-7} mol/L for 3 days elevated the protein expression levels of connexin 43 in both K562-ACR-TK cells and THP1-DXR-TK cells. This indicates that ATRA treatment upregulated expression of Connexin 43 in chemoresistant leukemic cells. Moreover, exposure of these cells to ATRA for 3 days resulted in a mild but significant decrease in cell survival rates, compared to the groups treated with GCV or ATRA alone, when the percentage of HSV-TK expressing cells varied among 10-60% for K562-ACR-TK cells and THP1-DXR-TK cells, respectively (Fig. 3B). These data suggested that use of ATRA upregulated connexin43 expression and augmented the bystander killing effect of HSV-TK/GCV.

VSV-G potentiated the killing by HSV-TK/GCV through enhancing bystander effect

Since use of ATRA affects the bystander effect of HSV-TK/GCV to only a mild extent, we next explored whether introduction of VSV-G could contribute to a more potent killing. To do so, we generated cells stably transduced with VSV-G by selection for GFP positivity. The VSV-G expression was evidently detected in the stable transductants K562-ACR-VSVG and THP1-DXR-VSVG by western blotting, as shown in Fig. 4A. Moreover, VSV-G mediated cell fustion was examined by morphological study, and we observed 6 ± 1.4% syncytia formed in VSV-G expressing cells, while ~0% in non-VSV-G expressing cells. To test whether expression of VSV-G impacts the bystander effect of HSV-TK/GCV, we co-cultured cells expressing HSV-TK with those expressing VSV-G at various ratios. As presented in Fig. 4B, we found that co-culture with VSV-G expressing cells resulted in a significant decrease in survival of K562-ACR-TK or THP1-DXR-TK cells, compared with control cells. These results indicate that co-culture of VSV-G expressing with TK-expressing resistant cells increased leukemic cell fusion and enhanced the bystander killing effect HSV-TK/GCV.

Combined use of VSV-G plus ATRA increased antileukemic effect of HSV-TK/GCV

We further tested the effect of combinatory use of VSV-G and ATRA. We found that, in K562-ACR-TK and THP1-DXR-TK cells, treatment with ATRA plus VSV-G gave rise to an increased percent (about 24-31% higher than control cells) of apoptotic and/or necrotic cells (Fig. 5), indicating that incorporation of VSV-G and ATRA into HSV-TK/GCV system produced more pronounced antileukemia effect. These data suggest that HSV-TK/GCV in combination with VSV-G and ATRA may represent a therapeutic strategy for chemoresistant AML.

DISCUSSION

Resistance to therapy is responsible for the poor outcomes of patients with AML. Suicide gene therapy for cancer has been extensively investigated both in experimental and clinical settings. Here, we explored application of the most commonly used suicide system, both in experimental and clinical settings, HSV-TK/GCV, to treat chemoresistant AML. In order to improve the therapeutic activity of HSV-TK/GCV, we modified it by introduction of VSV-G and ATRA into the system, and found that chemoresistant leukemic cells were suscepible to HSV-TK/GCV killing. Interestingly, the sensitivity of resistant leukemia cells to HSV-TK/GCV could be further increased by use of VSV-G and/or ATRA.

It has been previously shown that HSV-TK gene therapy can be used to inhibit hematologic malignancies. Earlier, Takenaga *et al*. reported that HSV-TK gene transduction followed by GCV treatment had potential value in killing a murine lymphatic leukemia cell line (Takenaga *et al*., 1996). Hoggarth *et al*. found that EGFP-fused HSV-TK retain the ability to effectively ablate leukemic and solid cancer cells, providing a new version of antitumor agent as well as a strategy of controlled removal of modified cells (Hoggarth *et al*., 2004). Furthermore, the HSV-TK/GCV system demonstrated in vivo therapeutic effects for eliminating insertional mutagenesis-induced murine leukemia (Blumenthal *et al*., 2007). In an immunodeficient (NOD/SCID) mouse model, HIV vector-based delivery of HSV-TK was shown to have an inhibitory activity against human adult T-cell leukemia cells (Miyake *et al*., 2007). In this study, we applied suicide gene therapeutic approach to treat chemoresistant human AML cells, and found that both Ara-C resistant and doxorubicin-resistant leukemic cells could be effectively killed, suggesting that the HSV-TK/GCV system could

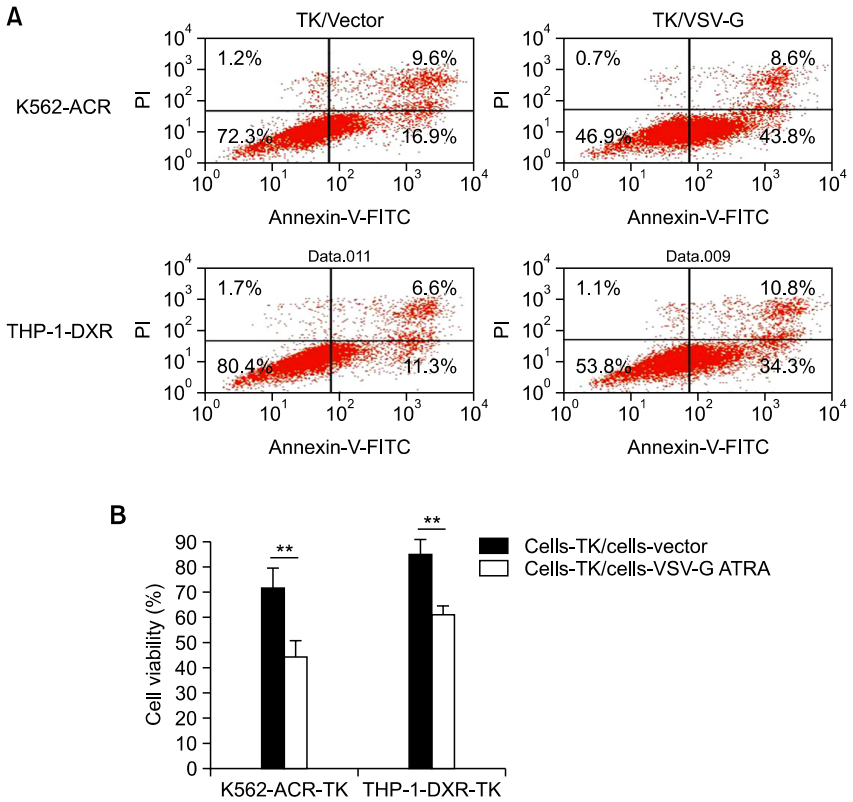

Fig. 5. Combinatory use of VSV-G plus ATRA increased anti-leukemic effect of HSV-TK/GCV. (A) HSV-TK expressing cells and VSV-G-expressing cells co-cultured at the ratio of 1:4 (for K562 and THP-1) were exposed to 100 μg/mL (for K562) or 10 μg/mL (for THP-1) GCV in the presence of 0.1 μM ATRA for 3 days, followed by cell apoptosis assay (as detailed in the Methods). Co-culture of HSV-TK expressing cells with empty vector-transduced cells at the same ratio was set as control. We found a significant increase in the percentage of mixed population containing TK-expressing and VSV-G-expressing cells treated with ATRA, as compared with controls. (B) Comparison of viability of HSV-TK expressing cells versus VSV-G-expressing cells. ***p*<0.01. Data shown here are representative of at least three experiments.

be used to directly target chemoresistant human AML cells.

Since the HSV-TK/GCV has limited clinical success due mainly to the low efficiency in transduction of tumor cells, modifications are needed to overcome this issue. It is known that the HSV-TK/GCV system typically produces the bystander effect, which is believed to be able to compensate for poor suicide gene delivery (Mesnil and Yamasaki, 2000; Van Dillen *et al*., 2002; Neschadim *et al*., 2012). The identity of the bystander effect signal and how it influences the nontargeted cells remains obscure. Among the mechanisms involved in this phenomenon, a large amount of works suggested that gap junctional intercellular communications play an important role in mediating the bystander effect of HSV-TK/GCV (Namba *et al*., 2001; Tanaka *et al*., 2001; Asklund *et al*., 2003; Matono *et al*., 2003; Robe *et al*., 2004). For example, gap junctional intercellular communication is shown to be involved in the transfer of the toxic metabolites of GCV, which pass directly from HSV TK-expressing cells to surrounding cells that do not express it (Mesnil and Yamasaki, 2000). Mesnil *et al*. reported that stimulation of intercellular communication of poor-communicating cells by gap-junction-competent cells enhances the HSV-TK/GCV bystander effect (Tanaka *et al*., 2001). Interestingly, Shirouzu *et al*. showed that bystander effect in suicide gene therapy is directly proportional to the degree of gap junctional intercellular communication in esophageal cancer (Matono *et al*., 2003). Modulation of the HSV-TK/GCV bystander effect in glioblastoma found a correlation with gap-junction intercellular communication (Robe *et al*., 2004). There is also evidence supporting gap junction-independent mechanism of the bystander killing (Drake *et al*., 2000; Grignet-Debrus *et al*., 2000; Chhipa and Bhat, 2007). Considering that many tumors, including many haematological ones, have a low number of gap junctions (Dilber and Gahrton, 2001), it is encouraging that ATRA, a commonly used antileukemia drug, was found to promote gap junctional intercellular communications through upregulation of Connexin 43 which is a major component of gap junctions (Park *et al*., 1997). In this work, we explored the combined use of ATRA and found for the first time that treatment with ATRA increased Connexin 43 expression and potentiated the bystander killing of HSV-TK/GCV. Consistently, enhancement of the bystander effect of suicide gene therapy by ATRA was also observed by other investigators in killing of solid cancer cells and human lens epithelial cells (Park *et al*., 1997; Chen *et al*., 2008; Li *et al*., 2011; Yang *et al*., 2012). These results point that ATRA treatment can augment the bystander killing of chemoresistant AML cells by HSV-TK/GCV.

In our previous experiments, we frequently observed that cells transfected to overexpress VSV-G protein tended to fuse with adjacent cells leading to death. This prompted us to explore the potential utility of VSV-G in the treatment of chemoresistant AML. Interestingly, when overexpressed in the resistant AML cells, VSV-G, either alone or in combination with ATRA,

enhanced HSV-TK/GCV killing, likely through increased cell fusion. In consistence, Galanis *et al*. earlier found that expression of two other viral FMGs could cause massive syncytial formation followed by death in transfected glioma cells, and markedly suppress their tumorigenicity in nude mice (Galanis *et al*., 2001). In addition, accumulating evidence indicate that the HIV envelope glycoprotein, which is another FMG, functions as a major mediator of the bystander cell apoptosis occurring during HIV pathogenesis, the mechanism of which involves the fusion/hemifusion activity of the HIV FMG (Garg and Blumenthal, 2008). These results are consistent that expression of VSV-G, as a potent promoter of cell fusion, can leads to cell death. Therefore, VSV-G can be used to increase the bystander antileukemic effect of HSV-TK/GCV on chemoresistant AML.

We realized that, although the efficacy of HSV-TK/GCV was increased by use of ATRA and VSV-G, the improvement was limited. There may be some rooms for further developments by, for example, replacement of the current version of VSV-G with a more potent pH value-sensitive mutant VSV-G (Zhu *et al*., 2013). In addition, switching to lentiviral pseudotyped vectors may achieve better therapeutic activities, since gene therapy using lentiviral manipulation of blood cells recently has obtained encouraging results (Aiuti *et al*., 2013; Biffi *et al*., 2013). Furthermore, combined use of ATRA and VSV-G enhanced suicide gene therapy with chemotherapeutics will likely produce desired effects (Neschadim *et al*., 2012).

The work we presented here demonstrated for the first time that suicide gene therapy, such as HSV-TK/GCV system, could be used to directly target chemoresistant human AML cells, and implied a potential value of VSV-G and ATRA in the enhancement of suicide gene therapy for resistant AML. Our findings may be suggestive of potential improvements in the treatment for malignancies other than chemoresistant AML.

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