Advances in Brief

Targeted Gene Delivery to Kaposi's Sarcoma Cells *via* the Fibroblast Growth Factor Receptor[1]

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Abstract

Kaposi's sarcoma (KS) is a major AIDS-related malignancy associated with significant morbidity and mortality. Current chemotherapeutic regimens are associated with a dismal prognosis. In an effort to develop a new approach to KS treatment, we devised a gene therapy-based adenovirus retargeting schema that redirects the adenovirus to fibroblast growth factor receptors endogenously present on the cell surface of KS cells. By using a bifunctional conjugate consisting of a blocking antiadenoviral knob Fab linked to basic fibroblast growth factor, FGF2, the gene transduction of KS cells was enhanced 7.7–44 fold; recombinant adenoviruses encoding either the firefly luciferase reporter gene, or the herpes simplex thymidine kinase gene, demonstrated quantitative enhancement of expression in the KS cell lines. In this regard, two KS cell lines that were previously refractory to native adenovirus transduction could be successfully transduced by the addition of the conjugate. This study thus addresses the utility of adenoviral retargeting to the FGF receptor in KS cells that are ordinarily transduction refractory to standardized approaches and allows practical development of gene therapy approaches for the treatment of human KS.

Introduction

Infection with HIV is associated with an increased incidence of a characteristic subset of neoplastic disorders, including KS[3] and non-Hodgkin's lymphoma (1). In this regard, KS is the major AIDS-associated malignancy and leads to significant morbidity (1, 2). Effective treatment for KS is currently lacking, with the duration of survival being only 9.9 months with some newer experimental protocols (3). Thus, the development of novel, more effective therapies is required for HIV-associated KS. To this end, a variety of gene therapy approaches have been developed for neoplastic diseases (4). Practical implementation of a gene therapy approach for KS would require efficient *in vivo* transduction of the tumor cells. Furthermore, some level of targeting to KS spindle cells would likewise be an important

criterion for vector selection.[4] This consideration is especially relevant in AIDS-related disseminated KS, because this tumor is thought to arise from vascular endothelial cells that are continuous with the systemic vasculature (2). Further complicating this endeavor, we have previously found that KS cells are refractory to transduction by a variety of viral and nonviral vector systems,[4] thus limiting even those gene therapy approaches based on locoregional gene delivery. To address this issue, we have developed a derivative vector possessing the capacity to target KS cells. In this report, we show that retargeting the adenovirus via the FGFR pathway allows extremely efficient transduction of KS cells. Such an approach may thus allow the translation of a toxin gene strategy for both locoregional and disseminated KS treatment.

Materials and Methods

Cell Lines. The human AIDS-KS cell line KSY-1 (5) was obtained from Dr. R. Gallo (University of Maryland, Baltimore, MD). The AIDS-KS cell line, RW376, and the classical KS cell line CVU-1, were obtained from Dr. P. Browning (Vanderbilt University, Nashville TN). KS-SLK (6) was derived from an oral KS lesion in an immunosuppressed patient and obtained from Dr. N. Popescu (NIH, Bethesda, MD).

All cell lines were grown in DMEM/Ham's F12 at a 1:1 ratio by weight (DMEM/F12, Cellgro Mediatech, Washington, DC) + 10% FBS (Hyclone Laboratories, Logan, UT) + 2 mM glutamine (Cellgro Mediatech) + penicillin/streptomycin (Cellgro Mediatech) at 37°C in 5% CO_2 (designated CM). Media changes were performed every 3–4 days. Cells were passaged using trypsin/EDTA (Cellgro Mediatech) when cells achieved confluency. Viability was determined in confluent cells exposed to trypsin/EDTA, centrifuged at 800 $\times$ g in the presence of CM, and counted using a hemocytometer after trypan blue exclusion. GCV (Cytovene) was purchased from Hoffmann Laboratories (Nutley, NJ). Tissue culture plates and flasks were manufactured by Nunclon (Denmark).

Recombinant Adenovirus. Recombinant E1A-deleted adenovirus (7) expressing firefly luciferase (AdCMVLuc) was provided generously by Dr. R. D. Gerard (University of Leuven, Leuven, Belgium). An E1-deleted Ad5 vector expressing the CMV-driven herpes simplex tk gene (AdCMVHSVtk) was constructed using homologous recombination techniques and has been reported previously (8). An E1-deleted recombinant adenovirus expressing an enhanced variant of green fluorescent protein (AdCAG-GFPS65T) was a generous gift from K. Moriyoshi and has been described previously (9). Recombinant adenoviruses were propagated on the permissive 293 cell line, purified using a cesium chloride gradient, and subsequently plaque titered on 293 cells using standard methods (10). Virus stocks were stored frozen at −80°C until use.

Fab-FGF2 Molecular Conjugate. The Fab-FGF2 conjugate was constructed by linking modified recombinant FGF2 (11) with the Fab fragment from a blocking monoclonal antibody (1D6.14) generated against the adenovirus serotype 5 knob region (12). For conjugation, the Fab was derivatized

with the heterobifunctional cross-linking reagent *N*-succinimidyl 3-[2-pyri-dyldithio]propionate (SPDP; Pharmacia, Uppsala, Sweden) at a 1:3 molar ratio and incubated at room temperature for 30 min to yield a modified Fab fragment (PDP-Fab). The PDP-Fab was dialyzed to remove unbound linker. Purified FGF2 was generated as described previously (11), reduced and mixed at a 2:1 molar ratio with PDP-Fab, and incubated at 4°C for 16 h with shaking. The conjugate was purified over a heparin-Sepharose column (Pharmacia) by loading in 10 mM Tris-HCl (pH 7.4), washing with 10 mM Tris-HCl/0.6 mM NaCl (pH 7.4), and eluting in 10 mM Tris-HCl/2 M NaCl (pH-7.4). The eluant was separated over a Sephacryl S-100 column equilibrated with Dulbecco's PBS (pH 7.4) to remove excess salt and unconjugated protein. The presence of PDP in the conjugate was confirmed by reducing an aliquot of the conjugate and measuring the absorbance of PDP (342 nm). The size and activity of the conjugate was subsequently analyzed by Western blot (13) and enzyme-linked immunoassay (14) analysis.

Adenovirus Infection Assays. To assess adenoviral transduction, 24,000 cells of each KS cell line were plated in triplicate into each well of a 12-well plate in the presence of 1 ml of CM. The cells were incubated overnight to allow cells to adhere. Infection complexes were mixed in a final volume of 50 μl containing: (*a*) adenovirus (AdCMVLuc or Ad-CAG-GFPS65T) at 50 pfu/cell, (*b*) adenovirus + Fab-FGF2 conjugate, (*c*) adenovirus + Fab, or (*d*) adenovirus + Fab-FGF2 conjugate + anti-FGF2 antisera (16 μl; Sigma Chemical Co.). The complexes were incubated in 1.5-ml polypropylene tubes at 27°C for 30 min. The mixtures were then diluted in DMEM/F12 + 2% FBS and added to each well in a volume of 200 μl. The cells were incubated at 37°C in 5% CO_2 for 1 h, then 800 μl of DMEM/F12 + 10% FBS were added to each well. Twenty-four h after the addition of virus, the cells were rinsed with PBS and assayed for luciferase activity or analyzed by FACS. For all luciferase assays, the cells were lysed in 200 μl of Promega (Madison, WI) lysis buffer. Twenty μl of each sample were subsequently mixed with 100 μl of Promega luciferase assay reagent according to the manufacturer's instructions, and triplicate determinations of duplicate samples were assayed in a Berthold luminometer.

To assess AdCMVHSVtk-mediated killing, 1 × 10⁵ KSY-1 or KS-SLK cells were plated in duplicate in six-well plates in 2 ml of CM. The cells were incubated at 37°C in 5% CO_2 overnight. The medium was aspirated, and infection mixtures containing 5 pfus/cell of either (*a*) AdCMVHSVtk, (*b*) AdCMVHSVtk + Fab, or (*c*) AdCMVHSVtk + Fab-FGF2 conjugate were added to each well in a volume of 500 μl of DMEM/F12 + 2% FBS. After 1 h of incubation at 37°C in 5% CO_2, 1.5 ml of CM were added. The cells were incubated for an additional 24 h, and the medium was then aspirated and replaced with CM in the absence or presence of 20 μM GCV. The medium was changed after 3 days, and cell counting was performed in triplicate for each of the duplicate wells 6 days after exposure to adenovirus to assess tk/GCV-mediated killing.

Immunocytochemistry. KS cells (2 × 10⁴/well) were plated into replicate wells of a 24-well plate tissue culture plate in CM and incubated at 37°C in 5% CO_2 for 48 h. The cells were rinsed twice with PBS and fixed with 10% formaldehyde/PBS for 2 h. Cells were rinsed, and endogenous peroxidase was blocked with 1% H_2O_2/methanol for 30 min. The cells were then rinsed and blocked in 3% BSA (Fraction V, Boehringer Mannheim, Mannheim, Germany)/PBS for 1 h at 27°C. Rabbit anti-FGFR antiserum (FGFR-1 and FGFR-2 reactive; Upstate Biotechnologies, Inc., Lake Placid, NY) or control rabbit IgG (Vector Laboratories, Burlingame, CA) was diluted 1:400 in 3% BSA/PBS and allowed to incubate on cells for 1 h at 37°C. The cells were rinsed and stained with diaminobenzidine (Sigma Chemical Co.) using a Vectastain rabbit horse-radish peroxidase kit according to the manufacturer's instructions. The cells were rinsed and stored underwater until photomicrographs were taken.

Statistics. Comparison of individual conditions were assessed using the Students' *t* test for equal means using Statex 1.2 for Macintosh (Dinan Software, Clinton, IA).

Results and Discussion

Gene therapy approaches for KS will depend upon the ability to accomplish efficient gene delivery to tumor cells *in situ*.[4] In this regard, adenoviral vectors have been used for a variety of *in vivo* cancer gene therapy applications. For this application, adenoviral vectors have the advantage of systemic stability and high levels of

gene expression *in vivo* (8, 15). We thus initially explored the utility of adenoviral vectors in a panel of human KS cell lines. Prior to modifying the adenovirus so that it would selectively retarget KS cells, we examined its native transduction efficiency. In these experiments, two AIDS-KS cell lines (KSY-1 and RW376), one KS cell line from an immunosuppressed patient (KS-SLK), and one classical KS cell line (CVU-1) were used. In the first set of experiments, the adenoviral transduction of each cell line was determined by infecting each cell line with AdCMVLuc in the presence or absence of the antiadenovirus knob Fab (Fig. 1) and subsequently measuring luciferase activity 24 h after infection. Of the cell lines tested, KSY-1 and KS-SLK were poorly transducible by adenovirus, yielding <10⁶ RLUs per assay. The KS cell line CVU-1 was moderately transducible (1.83 × 10⁶ ± 1.15 × 10⁵ RLUs per assay), whereas the RW376 cell line was highly transducible, yielding luciferase readings of 2.88 × 10⁶ ± 5.4 × 10⁴ RLUs per assay. The luciferase activity obtained after transduction using AdCMVLuc correlated with FACS analysis data obtained from cells that were infected with AdCAG-GFPS65T. In this context, by FACS analysis of the KS cell lines transduced with 100 pfu per cell of AdCAG-GFPS65T, fewer than 1% of KSY-1 and KS-SLK cells were transducible. The CVU-1 and RW376 KS cell lines were significantly more transducible, yielding 12 and 99% transduction efficiencies, respectively. In three cell lines, KSY-1, RW376, and KS-SLK, an antiadenoviral knob Fab blocked AdCMVLuc transduction by >50% ($P < 0.01$). The CVU-1 cell line exhibited a less dramatic (20%), albeit statistically significant ($P < 0.05$), block in adenoviral transduction. This low level of inhibition correlates with the modest level of transduction efficiency by the native adenovirus. This suggests that the degree to which these cells are refractory inversely correlates with knob-dependent cell binding.

On the basis of this recognition, we wondered whether we might overcome this limitation to infection by exploiting other cellular entry pathways to achieve effective gene transfer for adenoviral vectors. In this regard, we have recently developed an immunological approach that allows retargeting of adenovirus vectors to heterologous cellular pathways (12). As an initial validating step in these studies, we sought
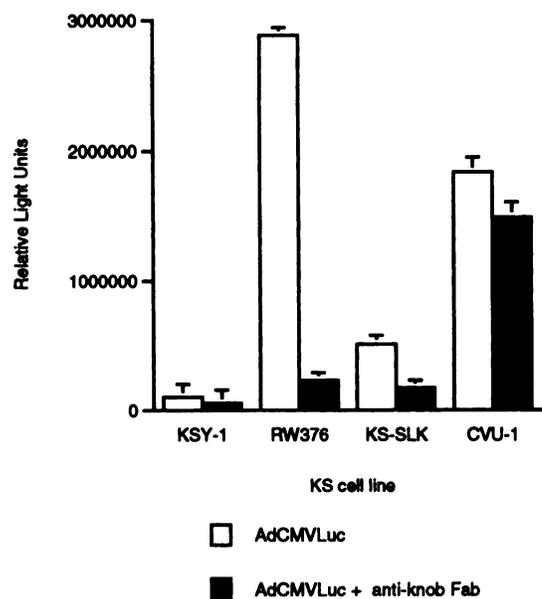

Fig. 1. Comparison of AdCMVLuc transduction for four KS cell lines. KS cells were incubated with recombinant adenovirus expressing luciferase in the absence (□) or presence (■) of a Fab fragment blocking adenoviral knob-mediated infection. Experiments were performed in triplicate. Data shown are means; *bars*, SD.
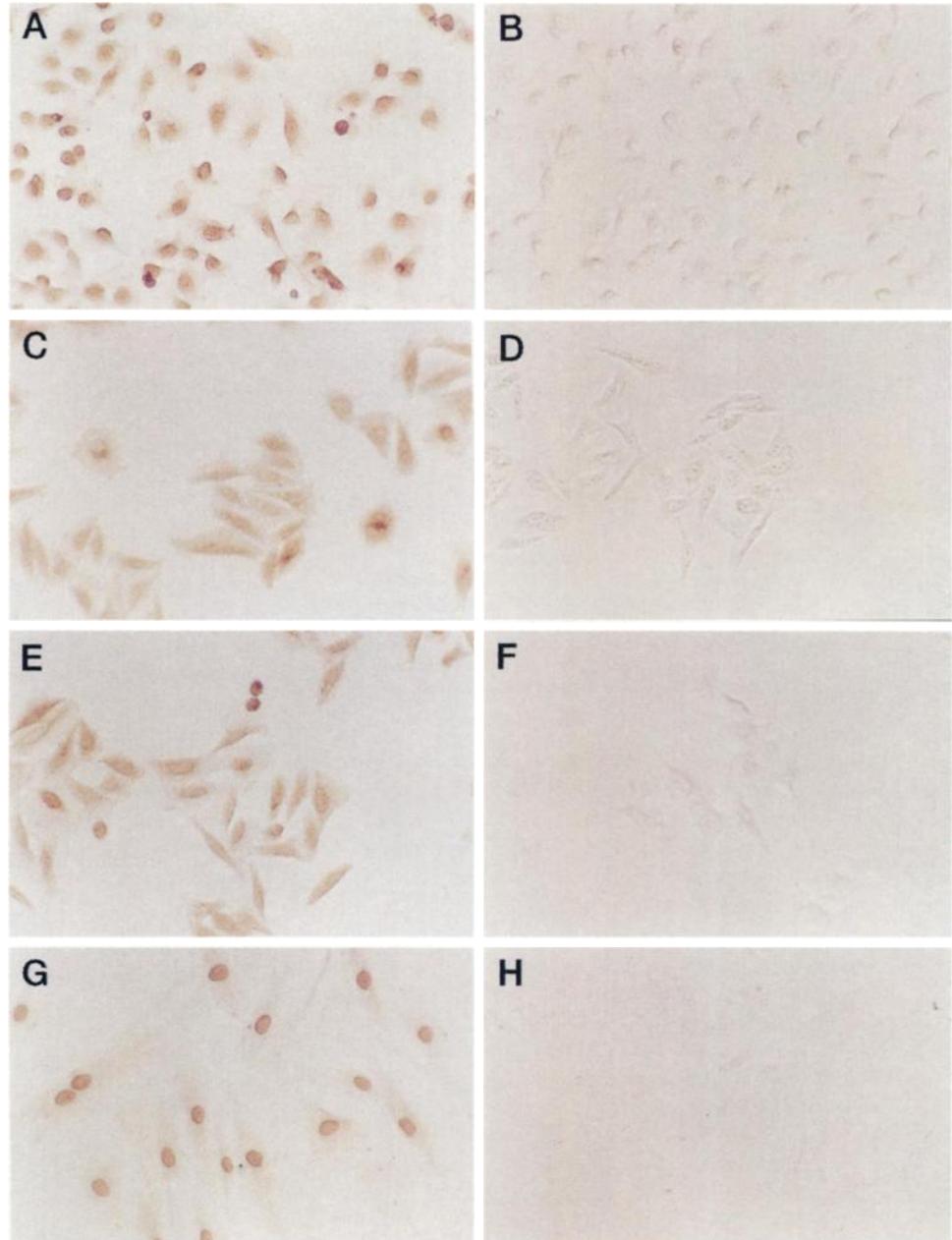
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Fig. 2. FGFR immunocytochemical reactivity of KS cell lines. KSY-1 (*A* and *B*), RW376 (*C* and *D*), KS-SLK (*E* and *F*), and CVU-1 (*G* and *H*) cell lines were stained with polyclonal antiserum raised against a peptide common to FGFR-1 and FGFR-2 (*A, C, E,* and *G*) or nonimmune control (*B, D, F,* and *H*).

to determine whether KS cells expressed FGFR, and whether this receptor could serve as a potential substrate for retargeting. As a first step, immunocytochemistry was performed on the four KS cell lines using a polyclonal antibody that simultaneously recognizes FGFR-1 and FGFR-2 via a common epitope. Immunoreactivity was observed in all four cell lines (Fig. 2) as well as in mouse fibroblasts (positive control; not shown). Distribution of immunoreactivity was predominantly nuclear, with scattered cell membrane staining in all four KS cell lines. The RW376 human KS cell line appeared to have the highest degree of membrane staining (Fig. 2C), whereas the CVU-1 KS line (Fig. 2G) had dense nuclear immunoreactivity. These studies demonstrate that FGFR is highly expressed in the relevant human KS cell lines and are thus consistent with previous reports (16).

Having established a biological rationale for our vector retargeting approach, we then tested the efficacy of FGFR-targeted adenovirus by using the KS cells as the cellular substrates. In the third set of experiments, we sought to determine whether we could immunolog-

ically retarget the adenovirus to FGFR using the Fab as a "handle" to the viral knob. To accomplish the retargeting between FGFR and the adenovirus-Fab complex, FGF2 was used as the targeting moiety because it binds with high affinity to both FGFR-1 and FGFR-2 and could be readily covalently conjugated to the Fab. To this end, a covalent conjugate was synthesized using SPDP to form a disulfide bond between the Fab and the cysteine present on modified FGF2. Western blot analysis confirmed that the majority of the Fab-FGF2 conjugate contained a single FGF2 molecule and a single Fab fragment. In addition, ELISA-based binding studies confirmed that the conjugate simultaneously retained knob-binding activity *and* FGF2 immunoreactivity (not shown).

To assess whether the Fab-FGF2 conjugate could retarget the adenovirus to KS cells, the conjugate was first preincubated with AdCMVLuc prior to cellular transduction. In an additional reaction mixture, the AdCMVLuc + Fab-FGF2 mixture was incubated further with blocking antisera raised against FGF2 to assess whether retar-
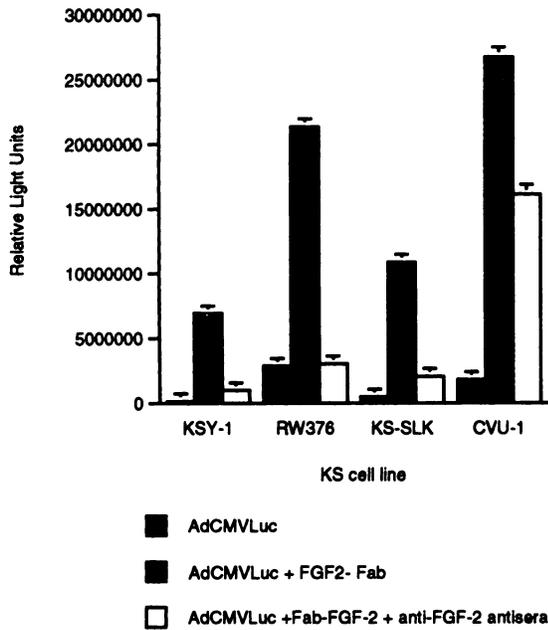
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Fig. 3. Enhanced AdCMVLuc infectivity of KS cell lines by Fab-FGF2 conjugate. The enhanced infectivity of the adenovirus-conjugate complex was assessed in the presence and absence of anti-FGF2 antisera. Data shown are means; *bars*, SD.

geting was occurring via the FGF2 moiety of the Fab-FGF2 conjugate. Fig. 3 demonstrates the results of the AdCMVLuc retargeting experiments using the Fab-FGF2 conjugate and also shows the FGF2 blocking experiments. The figure demonstrates a dramatic enhancement of AdCMVLuc transduction in all four KS cell lines when the adenovirus was premixed with the Fab-FGF2 conjugate. This enhancement was statistically significant for all four cell lines ($P < 0.001$) and represents a 44-fold increase in transduction for the KSY-1 cells and a 7.7-fold increase for RW376 cells. Of note, addition of antisera raised against FGF2 blocked ($P < 0.01$) the ability of the Fab-FGF2 conjugate to enhance AdCMVLuc transduction in all four KS cell lines. The attenuation of conjugate-mediated adenovirus transduction by anti-FGF2 antisera confirmed that retargeting was occurring *via* the FGF2 portion of the conjugate.

The experiments accomplished up to this point demonstrated that the low transduction efficiency of the adenovirus accomplished in KS

cells could be overcome by retargeting the adenovirus with a chemical conjugate that directed the adenovirus to the FGFR pathway. The detection of increased luciferase activity confirmed that transgene expression had taken place. In an effort to confirm that this paradigm had utility in the context of a gene therapy approach whereby a toxin gene is introduced into KS cells, we performed a series of experiments using a recombinant adenovirus encoding the conditionally toxic gene product herpes simplex tk (AdCMVHSVtk). In this experiment, we chose the two cell lines that had demonstrated the highest resistance to adenoviral gene transfer, KSY-1 and KS-SLK. Dose-response killing curves for these two cell lines were generated using cells infected with various concentrations of AdCMVHSVtk (data not shown) and subsequently maintained in the presence or absence of GCV. These experiments demonstrated that both cell lines showed little evidence of cell killing when cells were infected with 5 pfu/cell of AdCMVHSVtk in the presence of GCV. In the following experiment, we sought to potentiate AdCMVHSVtk gene transduction and subsequent sensitization to GCV in KS cells by addition of the Fab-FGF2 conjugate. In the experimental design, cells were treated with 5 pfus of either AdCMVHSVtk or AdCMVHSVtk complexed to Fab-FGF2. GCV-mediated killing was assessed by maintaining cells in the presence or absence of GCV. The results of these experiments are depicted in Fig. 4. Cell killing is expressed as a ratio of cells surviving in the presence of GCV:cells surviving in the absence of GCV. The figure demonstrates that retargeting AdCMVHSVtk with Fab-FGF2 resulted in a significant enhancement of the KS cells' susceptibility to GCV-mediated killing. These studies thus confirm our hypothesis that efficient gene transfer may be accomplished in KS cells by retargeting adenovirus via FGFR. Importantly, this maneuver not only increased transduction efficiency by quantitative levels, this effect was noted in all cell lines tested, even in the two cell lines that were essentially nontransducible by native adenovirus.

Acknowledgments

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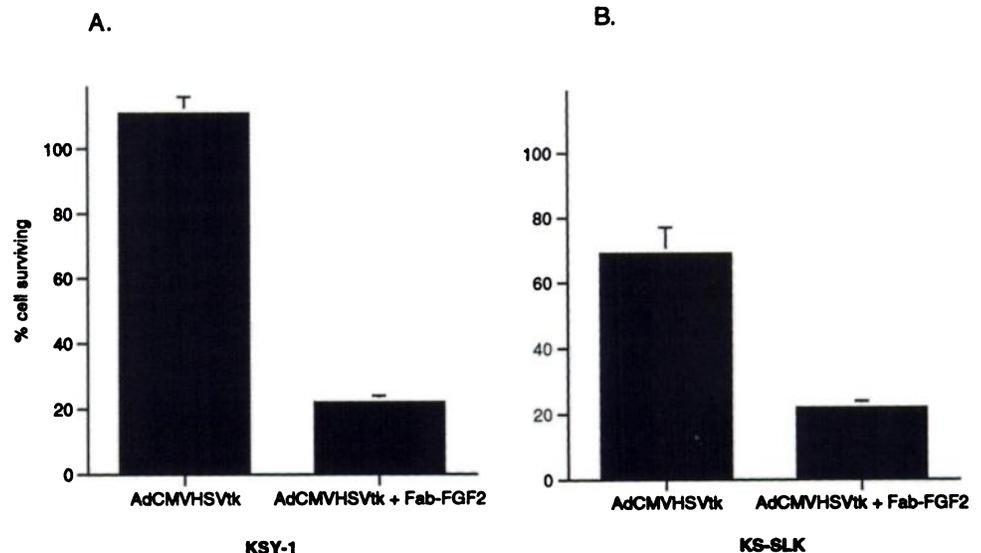
A.

KSY-1

B.

KS-SLK

Fig. 4. Enhanced AdCMVHSVtk/GCV cell killing in KSY-1 and KS-SLK cells by Fab-FGF2 conjugate. The effect of GCV on AdCMVHSVtk-transfected cells was assessed in the presence or absence of the conjugate and expressed as a percentage of cells surviving compared to the cells not exposed to GCV. Viable cells in duplicate wells were counted, in triplicate, after trypan blue exclusion. Data shown are means; *bars*, SD.

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