Integrin antagonist augments the therapeutic effect of adenovirus-mediated REIC/Dkk-3 gene therapy for malignant glioma

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Abstract

Reduced expression in immortalized cells/Dickkopf-3 (REIC/Dkk-3) was identified as a gene whose expression is reduced in many human cancers. REIC/Dkk-3 expression is also down-regulated in malignant glioma and regulates cell growth through caspase-dependent apoptosis. cRGD (EMD121974), an antagonist of integrins, has demonstrated preclinical efficacy against malignant glioma. In this study, we investigated the anti-glioma effect of combination therapy using an adenovirus vector carrying REIC/Dkk-3 (Ad-REIC) and cRGD. QRT-PCR revealed the reduction of REIC/Dkk-3 mRNA levels in malignant glioma cell lines. The reduction of REIC/Dkk-3 protein expression in malignant glioma cell lines was also confirmed with western blot analysis. After treatment with Ad-REIC and cRGD, the proliferative rate of malignant glioma cells was significantly reduced in a time-dependent manner. *In vivo*, there was a statistically significant increase in the survival of mice treated with Ad-REIC and cRGD combination therapy compared to Ad-REIC monotherapy. We identified an apoptotic effect following monotherapy with Ad-REIC. Moreover, cRGD augmented the anti-glioma efficacy of Ad-REIC. These results may lead to a promising new approach for the treatment of malignant glioma.

Introduction

Malignant gliomas remain challenging tumors to treat, and a variety of experimental therapies have failed to show effectiveness in clinical trials [1]. The median survival of aggressively treated patients with glioblastoma is approximately 14.6 months [2]. The resistance of gliomas to the conventional therapeutic regimen of surgery, radiotherapy, and chemotherapy has prompted many investigators to seek novel therapeutic approaches for this fatal disease [3].

Reduced expression in immortalized cells/Dickkopf-3(REIC/Dkk-3) was identified as a gene whose expression is reduced in many human cancers. REIC/Dkk-3 plays a role as a suppressor of the growth of several human cancers [4, 5]. REIC/Dkk-3 also regulates the growth and survival of glioma cells by caspase-dependent and -independent mechanisms via modification of the Wnt signaling pathway [4]. The Wnt signaling pathway regulates cell survival, at least in part, through inhibiting the proteasomal proteolysis of β-catenin [6]. β-Catenin protein levels are reduced in a time-dependent manner inversely with the increased expression of REIC/Dkk-3 [4].

Integrins are expressed in tumor cells and tumor endothelial cells [7-9], and they play important roles in angiogenesis and invasion in glioma [10-12]. αvβ3 and αvβ5 integrins regulate cell adhesion [13, 14], and antagonists of these integrins suppress tumor growth in certain preclinical models [15]. Several preclinical studies have shown an enhanced antitumor effect of

cRGD, an antagonist of these integrins, when it is administered in combinatorial therapeutic regimens [16-21]. Mikkelsen et al. demonstrated that cRGD dramatically amplified the efficacy of radiation therapy in an animal glioma model [22]. Previously, we demonstrated the enhanced therapeutic efficacy of oncolytic viruses on experimental glioma in combination with cRGD [23-25].

In the present study, we infected primary malignant glioma cells with the REIC/Dkk-3 gene using an adenovirus vector (Ad-REIC). We found that, in malignant glioma, REIC/Dkk-3 expression was down-regulated and that the gene regulates cell growth through caspase-dependent apoptosis and a reduction in β-catenin expression. We investigated the therapeutic effect of Ad-REIC in a malignant glioma xenograft model by intratumoral injection. Moreover, we showed the anti-glioma effect of combination therapy with Ad-REIC and the integrin antagonist cRGD.

Results

mRNA and protein expression of REIC/Dkk-3 in malignant glioma cell lines and normal human astrocytes

REIC/Dkk-3 mRNA expression was determined in normal human astrocyte (NHA) cells and the malignant glioma cell lines U251, Gli36Δ5, U87ΔEGFR using quantitative real-time

reverse-transcription PCR (QRT-PCR). The expression levels of REIC/Dkk-3 mRNA are displayed as a ratio between the target gene and a reference gene (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) to correct for variation in the amounts of RNA. The relative target gene expression level was normalized to the value from NHA cells (value = 1.0). REIC/Dkk-3 mRNA levels were lower in the malignant glioma cell lines than in NHA cells (relative quantification in U87ΔEGFR, U251, and Gli36Δ5: 0.00017, 0.073, and 0.374, respectively) (Figure 1A). The expression of REIC/Dkk-3 in malignant glioma cells was determined using western blot analysis, and protein band density was calculated using ImageJ software. REIC/Dkk-3 protein was detected as two major bands of ~60 and ~68 kDa. REIC/Dkk-3 expression was reduced in the malignant glioma cell lines compared with NHA cells, taking the REIC/Dkk-3 protein level in NHA cells as 1.0. The protein levels of REIC/Dkk-3 were as follows: U87ΔEGFR cells, 0.009; U251 cells, 0.106; Gli36Δ5 cells, 0.235; $P < 0.01$ (Figure 1B, C).

Overexpression of REIC/Dkk-3 protein with Ad-REIC

To examine the possible use of REIC/Dkk-3 as a tool for targeted gene-based therapy, we overexpressed REIC/Dkk-3 using Ad-REIC under the control of the CAG promoter [5]. An adenoviral vector carrying the LacZ gene with a CAG promoter (Ad-LacZ) was used as the

control vector. These adenoviral vectors were generated using replication-defective adenoviruses of serotype 5. REIC/Dkk-3 protein levels in U87ΔEGFR glioma cells at 36 h after treatment with Ad-REIC at a multiplicity of infection (MOI) of 10 were increased (Supplementary figure 1) ($P < 0.001$). The expression levels of REIC/Dkk-3 protein in U87ΔEGFR glioma cells at 36 h after treatment with Ad-REIC (MOI 10) and cRGD (0.1 μM) were increased as well as in cells treated with Ad-REIC (MOI 10) alone.

Expression of integrin αvβ3 and αvβ5 in cells treated with cRGD

U87ΔEGFR cells were treated with the indicated concentration of cRGD for 24 h and stained by immunofluorescence to assess integrin αvβ3 and αvβ5 expression, which was analyzed by flow cytometry. The initial expression levels of integrin αvβ3 and αvβ5 were remarkably high, while their expression in the presence of cRGD was slightly decreased at 1.0 μM and 10 μM in a dose-dependent manner (Figure 2).

Effect of cRGD on adenovirus infection and gene expression

Viral titers were calculated with the use of an Adeno-X qPCR Titration Kit (Clontech Laboratories, Mountain View, CA, USA) . The viral titer of the infected cells with Ad-LacZ was decreased when cRGD was added at 3 h before treatment with Ad-LacZ (Figure 3A). Moreover,

the adenovirus-mediated gene expression of LacZ in U87ΔEGFR cells was visualized by staining with X-gal. The expression levels of LacZ were significantly decreased when cRGD was added at 3 h before treatment with Ad-LacZ (Figure 3Ba–e).

Cytotoxic effect of Ad-REIC and combination treatment with cRGD

On the basis of the results shown above, to avoid cRGD interfering with viral infection, we utilized the following protocol: initially, glioma cells were infected with adenovirus, the media containing adenovirus were aspirated at 3 h after infection, and the cells were then incubated in fresh media containing cRGD at different concentrations. The cytotoxic effect of Ad-REIC and cRGD on glioma cells was investigated *in vitro*. The glioma cell lines were incubated with cRGD (0.1 or 1.0 μM) and Ad-REIC or Ad-LacZ at an MOI of 10 for the indicated time. The proliferation rate of the malignant glioma cells was decreased by treatment with Ad-REIC or cRGD in a time-dependent manner (Figure 4A, B). The combination of Ad-REIC and cRGD decreased the proliferation rate of the cells more than monotherapy. Moreover, to assess the effect of this combination, the cells were infected Ad-REIC at an MOI of 0 or 10 and subsequently treated with cRGD at a concentration of 0 or 0.1 μM. At 72 h after treatment with Ad-REIC and cRGD, the combination index (CI) in U87ΔEGFR cells was 0.80 and the CI in Gli36Δ5 cells was 0.87. This result indicated that the combination of Ad-REIC and cRGD

induced synergistic cytotoxicity.

Apoptotic effect of Ad-REIC and cRGD combination treatment

To confirm the apoptosis of the floating glioma cells treated with Ad-REIC and cRGD, the cells were stained using an *In Situ* Cell Death Detection Kit. Originally, U87ΔEGFR cells in culture were composed of bipolar cells; however, they became spherical and agglutinated when Ad-REIC and cRGD were added to the culture medium. In addition, Gli36Δ5 cells fused together when Ad-REIC and cRGD were added (Figure 5). Some of these deformed cells detached from the plate; these cells were stained by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method at 36 h after treatment with Ad-REIC and cRGD. As shown in Figure 6, TUNEL-positive cells were observed in the glioma cell lines (U87ΔEGFR and Gli36Δ5), but not in the NHA cells. A significant increase of TUNEL-positive cells was observed in the cells treated with Ad-REIC and cRGD monotherapy compared with Ad-LacZ ($P < 0.001$). Moreover, there was a significant increase in TUNEL-positive cells following combination therapy compared to Ad-REIC monotherapy, cRGD monotherapy, and control cells (Gli36Δ5: 47.7%, 10.4%, 13.0%, and 1.0%, respectively; all $P < 0.001$. U87ΔEGFR: 49.1%, 6.4%, 5.6%, and 0.6%, respectively; all $P < 0.001$).

Caspase expression and β-catenin degradation by REIC/Dkk-3 in malignant glioma cells

U87ΔEGFR glioma cells were treated with Ad-LacZ at an MOI of 10, Ad-LacZ and cRGD (0.1 μM), Ad-REIC at an MOI of 10, and Ad-REIC and cRGD combined. At 36 h after infection with Ad-REIC and at 33 h after adding cRGD or medium, glioma cells were harvested and the activity of caspase-8 and -9 was evaluated. In U87ΔEGFR cells, cRGD, Ad-REIC, and combination treatment induced the activated form of caspase-8. The cleaved form of caspase-9 expression was also increased in cells treated with Ad-REIC as well as in cells treated with Ad-REIC and cRGD combined (Figure 7).

Additionally, the Wnt signaling pathway regulates cell survival, at least in part, through inhibiting the proteasomal proteolysis of β-catenin. Therefore, we assessed its role on the expression of β-catenin in malignant glioma cells. β-Catenin protein levels were increased in the nucleus in a dose-dependent manner in U87ΔEGFR cells treated with cRGD. Conversely, β-catenin protein levels were reduced by Ad-REIC. Moreover, these levels decreased following combination treatment with cRGD and Ad-REIC (Figure 8).

Effect of cRGD treatment on the therapeutic efficacy of Ad-REIC in a xenograft mouse model

The antitumor effect of Ad-REIC, cRGD, and Ad-REIC and cRGD combined was tested in mice

bearing intracerebral glioma (U87ΔEGFR). Kaplan-Meier survival curves were used to assess the survival time of the U87ΔEGFR mouse glioma model treated with Ad-LacZ, Ad-REIC, or Ad-REIC and cRGD combined. When we injected 2.0×10^8 plaque-forming units (pfu) of the adenovirus, the survival time of mice treated with Ad-REIC was significantly longer than that of those treated with Ad-LacZ (median survival = 32 and 24 days, respectively; P = 0.0072; Figure 9A). In the next protocol, we reduced the amount of the adenovirus to 2.0×10^7 pfu. The survival time of mice treated with Ad-REIC and cRGD combined was significantly longer than that of those treated with Ad-LacZ or Ad-REIC alone (median survival = 25, 19, and 19 days, respectively; P = 0.0002; Figure 9B). An increased number of apoptotic cells was observed using the TUNEL assay in tumors treated with Ad-REIC and cRGD combined compared with Ad-REIC and control tumors (59.3%, 13.8%, and 2.2%, respectively; P < 0.0001; Figure 10).

Discussion

Our study indicated that the expression levels of REIC/Dkk-3 mRNA and protein were down-regulated in malignant glioma cell lines. The expression levels of REIC/Dkk-3 protein in U87ΔEGFR glioma cells after treatment with Ad-REIC and cRGD were increased as well as in cells treated with Ad-REIC alone. *In vitro*, after treatment with Ad-REIC and cRGD, the proliferation rate of malignant glioma cells was significantly reduced in a time-dependent

manner. In malignant glioma cells, increased REIC/Dkk-3 expression led to a remarkable increase in the number of TUNEL-positive cells. TUNEL-positive cells were observed in malignant glioma cell lines, but not among NHA cells. *In vivo*, the survival rate of mice treated with Ad-REIC and cRGD combination therapy was significantly improved compared to single treatment. In addition, an increased number of apoptotic cells was revealed by the TUNEL assay in tumors treated with Ad-REIC and cRGD combined compared with Ad-REIC and control.

Effect of Ad-REIC on glioma *in vitro*

We found that the increased expression of REIC/Dkk-3 by using an adenovirus vector led to a remarkable increase in the number of TUNEL-positive cells. Mizobuchi et al. reported that the overexpression of REIC/Dkk-3 with a plasmid vector induced apoptosis in malignant glioma cells [4]. The *REIC/Dkk-3* gene regulates cell growth through caspase-dependent apoptosis, especially via caspase-9. Our data also showed that the activated form of caspase-9 in glioma cells treated with Ad-REIC was significantly higher than in those treated with control.

Moreover, β-catenin expression was decreased with the increased expression of REIC/Dkk-3. The activation of the Wnt signaling pathway is thought to contribute to the development of some human cancers, and β-catenin is considered a key element of the Wnt signaling pathway. Wnt signaling inhibits the release of cytochrome C and the subsequent

activation of caspase-9 induced by apoptotic stimuli [26].

Advantage of combination therapy with cRGD

Our results suggested that cRGD augmented the anti-glioma efficacy of Ad-REIC. Some reports have indicated the efficacy of combination therapy with cRGD. It has been used in combination with temozolomide against murine melanoma cells [27], with radiotherapy against human prostate cancer cells and human glioma cells [28], and with an oncolytic virus against rat and human glioma cells [23, 24]. Similar to these reports, we found that cRGD augmented the anti-glioma efficacy of Ad-REIC.

Previously, we showed that cRGD inhibited integrin binding and activated caspase-8 through an extrinsic pathway [21]. This caspase-8 activation effect of cRGD would enhance the effect of other cytotoxic therapies. In addition, Ad-REIC regulated cell growth through caspase-dependent apoptosis, mainly via caspase-9. The combination of the intrinsic pathway with caspase-9 activation and the extrinsic pathway with caspase-8 activation, is more efficient for inducing apoptosis than a single pathway [23, 24].

For the upstream pathway, β-catenin protein levels were increased in the nucleus in a dose-dependent manner in glioma cells treated with cRGD. cRGD treatment reportedly induces the dissociation of cadherin from β-catenin [29] and, upon Wnt stimulation, results in an increase

in the “free” (uncomplexed to cadherin) cytosolic levels of β-catenin. This leads to its subsequent accumulation within the nucleus [30, 31]. This indicates that cRGD accelerated the Wnt/β-catenin signaling pathway. Conversely, monotherapy with Ad-REIC decreased β-catenin expression. The suppression of Wnt signaling via Ad-REIC induced β-catenin degradation [4], preventing its translocation to the nucleus. Moreover, combination treatment of cRGD with Ad-REIC reduced the intra-nuclear levels of β-catenin protein more than Ad-REIC alone. As a result, cRGD could sensitize glioma cells to Ad-REIC treatment by increasing Wnt/β-catenin signaling.

Efficacy of Ad-REIC on glioma *in vivo*

From our results, the mice that received a single treatment of Ad-REIC (2.0×10^8 pfu) had significantly prolonged survival compared to the Ad-LacZ-treated mice. This amount of virus was chosen in reference to previous reports. For malignant mesothelioma, 4.0×10^8 pfu of Ad-REIC were injected [32]. For gastric cancer, the mice were injected intraperitoneally with 1.0×10^9 pfu of Ad-REIC in 4 mL phosphate-buffered saline (PBS) [33]. For prostate cancer, 1.2×10^8 pfu of an adenovirus vector carrying mouse REIC/Dkk-3 cDNA were injected intratumorally [34]. Interestingly, the survival rate of mice treated with Ad-REIC and cRGD combination therapy was significantly improved compared to single treatment with Ad-REIC.

We could decrease the amount of virus from 2.0×10^8 pfu to 2.0×10^7 pfu by combination treatment.

As a limitation of this study, in the *in vivo* experiments, there are not only tumor cells, but also other cell types, such as endothelial cells, endogenous astrocytes, and glial cells, that could express αvβ3/αvβ5. The effect of cRGD may not exclusively be owing to its effects on the injected tumor cells. Further studies are needed to elucidate this mechanism.

Future directions

We are developing Ad-REIC for use in clinical trials. The first-in-human, phase I/IIa clinical study of *in situ* Ad-REIC gene therapy for prostate cancer is being conducted at Okayama University Hospital. If the safety and efficacy of this therapy is proven, this might help us to perform a clinical trial for glioblastoma treatment with Ad-REIC. Certainly, as for cRGD, in the CENTRIC study (phase III), which compares the efficacy and tolerability of cRGD in patients with newly diagnosed glioblastoma and a methylated O6-methylguanine-DNA methyltransferase (*MGMT*) gene promoter status, cRGD failed to prolong progression-free survival or overall survival in patients with newly diagnosed glioblastoma and a methylated *MGMT* gene promoter. However, this point warrants a more detailed analysis with variation of the dosage of cRGD and Ad-REIC. Furthermore, we should also evaluate combination therapy

of Ad-REIC with other drugs such as temozolomide or bevacizumab.

In conclusion, the overexpression of REIC/Dkk-3 induced apoptotic cell death in malignant glioma cell lines. Moreover, we revealed that cRGD augments the anti-glioma efficacy of Ad-REIC.

Materials and methods

Cell lines and reagents

The glioma cell lines U87ΔEGFR, U251, and Gli36Δ5 were seeded on tissue culture dishes (BD Falcon, Franklin Lakes, NJ, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U penicillin, and 0.1 mg/mL streptomycin. NHA cells were purchased from Takara Bio, Inc. (Shiga, Japan).

cRGD was generously provided by Merck KGaA (Darmstadt, Germany) and the Cancer Therapy Evaluation Program, National Cancer Institute, National Institutes of Health.

Adenovirus vector carrying REIC/Dkk-3

For Ad-REIC under the control of the CAG promoter (CMV early enhancer/chicken β-actin promoter), the full-length human REIC/Dkk-3 gene was inserted into the cosmid vector pAxCAwt and then transferred into an adenoviral vector using the COS-TPC method (Takara

Bio, Inc., Shiga, Japan) [5]. An adenoviral vector carrying the LacZ gene with a CAG promoter (Ad-LacZ) was used as the control vector. These adenoviral vectors were generated using replication-defective adenoviruses of serotype 5.

QRT-PCR

Total RNA was isolated from cultured glioma and NHA cells using an RNeasy® Mini Kit (QIAGEN, Hilden, Germany), and was reverse transcribed with oligo-dT primers using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers specific for each gene were designed using Primer 3 Plus Software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesized by Invitrogen. The resulting cDNA was amplified by PCR using gene-specific primers and the StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and a QuantiTect™ SYBR® Green PCR Kit (QIAGEN, Hilden, Germany). A log-linear relationship between the amplification curve and quantity of cDNA in the range of 1–1000 copies was observed. The cycle number at the threshold was used as the threshold cycle (Ct). mRNA expression levels were determined from 2-ΔΔCt using StepOne Software (version 2.2; Applied Biosystems, Foster City, CA, USA). The amount of cDNA in each sample was normalized to

the crossing point of the housekeeping gene GAPDH. The following thermal cycling parameters were used: denaturation at 95°C for 10 min followed by 45 cycles at 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s. The relative mRNA upregulation of each gene was calculated using their respective crossing points with the following formula, as described previously [24]: REIC/DKK-3 (forward) 5′-GTAAGTTCCCCTCTGGCTTG-3′, REIC/Dkk-3 (reverse) 5′-AAGCACCAGACTGTGAAGCCT-3′, GAPDH (forward) 5′-GGGTGTGAACCATGAGAAGTATGA-3′, GAPDH (reverse) 5′-TGCTAAGCAGTTGGTGGTGC-3′.

Viral titration assay

PCR was performed employing an Adeno-X qPCR Titration Kit (Clontech Laboratories, Mountain View, CA, USA) and using a StepOnePlus Real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The forward and reverse primers were provided in the kit. The PCR cycling conditions were 95°C for 30 s, followed by 95°C for 3 s and 60°C for 25 s for 40 cycles, and then 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The DNA copy number was determined from a standard curve generated by plotting the Ct values of the diluted Adeno-X DNA Control Template against their respective copy numbers, according to the manufacturer's instructions.

LacZ staining

Ad-LacZ-infected cells were detected using 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining (Wako, Osaka, Japan). Suspended cells were attached to slides via Cytospin (Shandon Cytospin® Centrifuge; Thermo Scientific, Waltham, MA, USA). The cell were fixed in 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 15 min, and then incubated overnight at 37°C in an X-gal solution. The cells were counted under a light microscope.

Flow cytometry

To measure the expression levels of integrin, U87ΔEGFR and Gli36Δ5 cells were exposed to a fluorescein isothiocyanate(FITC) -conjugated mouse anti-human integrin αvβ3 antibody (eBioscience, San Diego, CA, USA) and a phycoerythrin(PE)-conjugated mouse anti-human integrin αvβ5 antibody (R&D Systems, Minneapolis, MN, USA). Mouse IgG1 K isotype control FITC (eBioscience) and mouse IgG1 isotype control PE (R&D Systems) were used as controls. The samples were run on a flow cytometer (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using CellQuest software.

Cytotoxicity assay

The cells were cultured in a flat-bottomed 6-well dish at a concentration of 4.0×10^5 cells per

well. The cells were infected with Ad-REIC or Ad-LacZ at an MOI of 10. The media containing adenovirus were aspirated at 3 h after infection, and the cells were then incubated in fresh media containing cRGD at a concentration of 0.1 μM for U87ΔEGFR and 1 μM for Gli36Δ5. At 48, 72, and 96 h later, the viability of the cells was examined. To determine the number of the cells attached to the bottom of each culture well, we used a Z2™ COULTER COUNTER® (Beckman Coulter, Brea, CA, USA) and counted the cells in 3 different wells.

To assess the combination effect of Ad-REIC and cRGD, the cells were infected Ad-REIC at an MOI of 0 or 10, the media containing adenovirus were aspirated at 3 h after infection, and subsequently treated with cRGD at a concentration of 0 or 0.1 μM. The combination index (CI) of Ad-REIC with cRGD was calculated as (observed fraction value of combination group) / (expected fraction value of combination group), in which the expected fraction value of combination group were estimated as (observed mean value of Ad-REIC) / (mean control value) × (observed mean value of cRGD) / (mean control value). CI < 1, = 1, and > 1 represent synergistic, additive, and antagonistic effects, respectively[35].

Western blot analysis

The cells were cultured in a 100-mm dish (BD Falcon, Franklin Lakes, NJ, USA). Cell culture media were aspirated, the dishes were washed in PBS twice, and the cells were lysed in 1% SDS.

The lysates were sonicated for analysis of whole cell proteins. Nuclear proteins were isolated using an NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Extracted protein samples were separated by gel electrophoresis and transferred onto PVDF membranes. After blocking in 5% skim milk, the membranes were incubated overnight with primary antibodies at 4°C. The membranes were washed with Tris-buffered saline-Tween 20 (TBST), incubated with secondary antibodies at room temperature for 1 h, and rinsed with TBST. Signals were visualized using the ECL Prime Western Blotting Detection System (GE Healthcare Life Sciences, Buckinghamshire, UK). The primary antibodies mouse anti-human REIC/DKK-3 (provided by the Department of Urology, Okayama University, Okayama, Japan), β-catenin, caspase-8 and -9, and TATA-binding protein (TBP) (Cell Signaling Technology, Danvers, MA, USA) were diluted 1:1000 in TBST. The β-actin antibody (Sigma, St. Louis, MO, USA) was diluted 1:5000 in TBST. The secondary antibody was horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA), diluted 1:6000 (REIC/Dkk-3, β-actin) or 1:2000 (β-catenin, caspase-8 and -9, and TBP) in TBST.

Apoptosis assay

Apoptotic tumor cells were detected using an *In Situ* Cell Death Detection Kit (Roche,

Mannheim, Germany). Suspended cells were attached to slides via Cytospin (Shandon Cytospin® Centrifuge; Thermo Scientific, Waltham, MA, USA). The cells were fixed in 4% paraformaldehyde at room temperature for 60 min, incubated in 3% hydrogen peroxide in methanol for 10 min at room temperature to block endogenous peroxidase activity, and then permeabilized for 2 min on ice with a permeabilization buffer. The cells were incubated at 37°C for 60 min in a TUNEL reaction mixture. Apoptotic cells were counted under a fluorescent microscope. For one experiment, the apoptotic cells were counted in 7 different fields, and the average percentage of apoptotic cells was calculated.

***In vivo* experiments**

All experimental animals were housed and handled in accordance with the guidelines of the Okayama University Animal Research Committee. Before implantation, 85–90% confluent U87ΔEGFR cells were trypsinized and centrifuged at 100 × *g* for 5 min; the resulting pellet was resuspended in PBS, and the cell concentration was adjusted to 1.0×10^5 cells/μL. U87ΔEGFR cells (2 μL) were injected into 6-week-old BALB/c female nude mice (CLEA Japan, Inc., Tokyo, Japan). The mice were anesthetized and placed in stereotactic frames (Narishige, Tokyo, Japan) with their skulls exposed. Tumor cells were injected with a Hamilton syringe (Hamilton, Reno, NV, USA) into the right frontal lobe (3 mm lateral to the midline, 1 mm posterior to the coronal

suture, 3 mm depth from the dura) and the syringe was withdrawn slowly after 5 min to prevent reflux. The skulls were then cleaned, and the incision was sutured. Seven days following tumor inoculation, all mice bearing brain tumors were reanesthetized and subsequently injected with Ad-REIC and Ad-LacZ stereotactically at the tumor inoculation site using the same coordinates. cRGD and solvent were administered 3 times per week intraperitoneally at 200 μg/100 μL saline starting on Day 9 after tumor cell implantation.

TUNEL staining *in vivo*

An *In Situ* Cell Death Detection Kit, POD (Roche, Mannheim, Germany) was used for the TUNEL assay. The sections were deparaffinized and rehydrated. They were incubated in 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity. The sections were digested for 15 min using 20 μg/mL proteinase K, recombinant, PCR grade (Roche, Mannheim, Germany) in 10 mM Tris-HCl, pH 7.5. They were incubated with the TUNEL reaction mixture in a humidified chamber for 60 min at 37°C. They were then incubated with Converter-POD in a humidified chamber for 30 min at 37°C. The sections were lightly counter-stained with Mayer's hematoxylin and analyzed under a light microscope.

Statistical analysis

Data from the protein levels of REIC/Dkk-3, viral titration assay, and LacZ staining were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, and the percentage of TUNEL-positive cells in vitro and in vivo was analyzed using one-way ANOVA followed by the Games-Howell post hoc test. Kaplan-Meier curves were compared using the log-rank test. Statistical analysis was performed using SPSS statistical software (version 20; SPSS, Inc., Chicago, IL, USA). P-values < 0.05 were considered statistically significant.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

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Figure legends

Figure 1

REIC/Dkk-3 mRNA levels in NHA cells and glioblastoma cell lines (A) were determined by QRT-PCR and expressed as molar ratios to those of GAPDH. QRT-PCR revealed a reduction in REIC/Dkk-3 mRNA levels in malignant glioma cell lines (relative quantification: U87ΔEGFR, 0.00017; U251, 0.073; Gli36Δ5, 0.374; NHA, 1). (B, C) Western blot analysis revealed a reduction of REIC/Dkk-3 protein levels in malignant glioma cell lines compared to NHA cells (U87ΔEGFR, 0.009; U251, 0.106; Gli36Δ5, 0.235; NHA, 1). Protein band density was calculated using ImageJ software. Mean ± SD. * $P < 0.01$.

Figure 2

Expression levels of integrin αvβ3 and αvβ5. U87ΔEGFR cells were exposed to the indicated concentration (0, 1.0, or 10 μM) of cRGD for 24 h, stained by immunofluorescence for integrin αvβ3 (A) and αvβ5 (B) expression, and analyzed by flow cytometry. The initial expression levels of integrin αvβ3 (a) and αvβ5 (d) were remarkably high. cRGD at any concentration had little impact on both integrin αvβ3 (b, c) and αvβ5 (e, f) expression. Dotted lines: mouse IgG1 isotype controls.

Figure 3

Viral titer and LacZ expression under treatment with cRGD. (A) cRGD (0.1 or 1 μM) was added at 3 h before or at the same time as treatment with Ad-LacZ (MOI 10). After 3 h, viral titers were calculated using an Adeno-X qPCR Titration Kit (Clontech Laboratories, Mountain View, CA, USA). The viral titer of the infected cells with Ad-LacZ was decreased when cRGD was added at 3 h before treatment with Ad-LacZ. (B) The cells were incubated with media containing cRGD at a concentration of 0 or 0.1 μM for U87ΔEGFR. At 3 h before or 0 and 3 h later, the cells were infected with Ad-LacZ. The infection efficiency of Ad-LacZ to U87ΔEGFR cells was visualized by staining with X-gal. Each panel shows each time course: (a) adding cRGD at 3 h before viral infection, (b) at 0 h, (c) at 3 h after viral infection, (d) untreated with virus, and (e) Ad-LacZ alone. (C) Infection efficiency was significantly decreased when cRGD was added at 3 h before infection with Ad-LacZ. Bar = 200 μm. (A) Mean ± SD. * P = 0.001. **P = 0.002. (C) Mean ± SD. *P < 0.0001.

Figure 4

Cytotoxicity after treatment with Ad-REIC and cRGD. The cells were infected with Ad-REIC or Ad-LacZ at an MOI of 10. The media containing adenovirus were aspirated at 3 h after infection, and the cells were incubated in fresh media containing cRGD at a concentration of 0.1

μM for U87ΔEGFR and 1 μM for Gli36Δ5. At 48, 72, and 96 h later, the viability of the cells was examined. After treatment with Ad-REIC and cRGD, the proliferation rate of malignant glioma cells (A: U87ΔEGFR glioma cells, B: Gli36Δ5 glioma cells) was reduced compared to the other treatment groups in a time-dependent manner.

Figure 5

Most of the U87ΔEGFR cells treated with Ad-LacZ were composed of bipolar cells. However, they became spherical and agglutinated when they were treated with the combination of Ad-REIC (MOI = 10) and cRGD (0.1 μM) (top row, a-d). In addition, Gli36Δ5 cells fused together when they were treated with the combination of Ad-REIC (MOI = 10) and cRGD (1 μM) (bottom row, e-h). Bar = 100 μm.

Figure 6

The combination of Ad-REIC and cRGD increased the number of TUNEL-positive cells. The cells were infected with Ad-REIC or Ad-LacZ at an MOI of 10. The media containing adenovirus were aspirated at 3 h after infection, and the cells were incubated in fresh media containing cRGD at a concentration of 0.1 μM for U87ΔEGFR and 1 μM for Gli36Δ5. After 36 h, the cells were stained by the TUNEL method. Many TUNEL-positive cells were observed in

the glioma cell lines (A, B; Gli36Δ5 and C, D; U87ΔEGFR), but not in the NHA cells (E, F). A significant increase of TUNEL-positive cells was observed in the cells treated with Ad-REIC and cRGD monotherapy compared with Ad-LacZ. Moreover, there was a significant increase in TUNEL-positive cells treated with combination therapy compared to Ad-REIC single treatment, cRGD alone, and control cells (*P < 0.001). TUNEL-positive cells, green; DAPI (4′,6-diamidino-2-phenylindole; cell nuclei), blue. Bar = 100 μm. Mean ± SD.

Figure 7

Caspase-8 and -9 expression in U87ΔEGFR glioma cells after treatment with Ad-REIC and cRGD. U87ΔEGFR cells were infected with Ad-REIC or Ad-LacZ at an MOI of 10. At 3 h after infection, the cells were incubated in media containing cRGD at a concentration of 0.1 μM. Immunoblot analysis showed that cleaved caspase-8 was processed in the U87ΔEGFR cell line following treatment with cRGD and Ad-REIC. Cleaved caspase-9 was increased following treatment with Ad-REIC and combination treatment with cRGD.

Figure 8

Expression of β-catenin in the nucleus in U87ΔEGFR glioma cells after treatment with Ad-REIC and cRGD. (A) U87ΔEGFR cells were incubated with media containing cRGD at

different concentrations. β-Catenin expression was increased when U87ΔEGFR cells were treated with cRGD at a concentration of 0.01, 0.1 μM or more. (B) U87ΔEGFR cells were infected with Ad-REIC or Ad-LacZ at an MOI of 10. At 3 h after infection, the cells were incubated in media containing cRGD at a concentration of 0.1 μM. β-Catenin expression was decreased with the increased expression of REIC/Dkk-3. In addition, there was a significant decrease in the expression of β-catenin in cells treated with combination therapy compared with Ad-REIC monotherapy.

Figure 9

Kaplan-Meier survival curves of the U87ΔEGFR mouse glioma models treated with Ad-REIC or the combination of Ad-REIC and cRGD. (A) Seven days after tumor implantation, we treated the mice with Ad-REIC or Ad-LacZ (2.0×10^8 pfu) by direct intratumoral injection. The survival time of mice treated with Ad-REIC was significantly longer than those treated with Ad-LacZ (median survival = 32 and 24 days, respectively; P = 0.0072). (B) In the next protocol, we reduced the amount of adenovirus to 2.0×10^7 pfu. At 2 days after treatment with adenovirus, we started to administer either cRGD (200 μg/100 μL saline) or saline intraperitoneally 3 times per week. The survival time of mice treated with Ad-REIC and cRGD combined was significantly longer than that of those treated with Ad-LacZ or Ad-REIC alone (median survival

= 25, 19, and 19 days, respectively; P = 0.0002).

Figure 10

The mice were sacrificed at 3 weeks after treatment, and the brains treated with Ad-LacZ (2.0×10^7 pfu) (A), Ad-REIC (2.0×10^7 pfu) (B), and Ad-REIC (2.0×10^7 pfu) and cRGD combined (C) were analyzed using the TUNEL assay. An increased number of apoptotic cells (brown) in tumors treated with Ad-REIC and cRGD combined compared with Ad-REIC and control was observed. (**A**) Ad-LacZ, 2.2%; (**B**) Ad-REIC, 13.8%; (**C**) Ad-REIC and cRGD, 59.3%. Bar = 100 μm. Mean ± SD. *P = 0.0044, **P < 0.0001.

Supplementary figure 1

Expression levels of REIC/Dkk-3 protein in U87ΔEGFR glioma cells after treatment with Ad-REIC and cRGD. U87ΔEGFR cells were infected with Ad-REIC or Ad-LacZ at an MOI of 10. At 3 h after infection, the cells were incubated in media containing cRGD at a concentration of 0.1 μM. (A) The expression levels of REIC/Dkk-3 protein in U87ΔEGFR glioma cells after treatment with Ad-REIC and cRGD were increased as well as in cells treated with Ad-REIC alone. (B) Quantification of the expression ratio (average expression levels: Ad-LacZ, 1; Ad-LacZ+ cRGD, 1.205; Ad-REIC, 6.157; Ad-REIC+cRGD; 6.240). Protein band density was

calculated using ImageJ software. Mean ± SD. * $P < 0.001$.