Article

RGD-FasL Induces Apoptosis of Pituitary Adenoma Cells

Lukui Chen[1, 5], Guohong Zhuang[2], Wenzhu Li[3], Yunsheng Liu[4], Junqing Zhang[1] and Xinhua Tian[1]

This study was to investigate the cytotoxic effects on pituitary adenoma cell lines GH3/MMQ/AtT20 induced by RGD-FasL and the underlying mechanism. Fas/DcR3 mRNAs were detected by RT-PCR and their surface expressions were measured by flow cytometry. Cytotoxicity exerted by RGD-FasL on tumor cells was measured with MTT assay and the induced apoptosis was determined by agarose gel electrophoresis. The cell cycle and apoptosis was assessed by flow cytometry with PI staining. The expressions of caspase8/9/3, Bcl-2, RANKL and JNK2 were detected by Western blotting. Approximately 13.7% of GH3 cells, 25.5% of MMQ cells, 22.2% of AtT20 cells express Fas, while 23.9% of GH3 cells, 24.1% of MMQ cells, 4.6% of AtT20 cells express DcR3. The cytotoxic effects of FasL/RGD-FasL on tumor cells were all taken in a dose-dependent manner. Cell lines MMQ/AtT20 showed the same sensitivity to RGD-FasL as to FasL, while cell line GH3 was less sensitive to RGD-FasL. The cell cycle analysis indicated that RGD-FasL could inhibit cells in G0/G1 phase and G2/M phase. In MMQ and AtT20 cells treated with RGD-FasL, the AI was not significantly different from that treated with FasL, while in GH3 cells treated with RGD-FasL, the AI was lower than that treated with FasL. The expressions of caspase-8/9/3, RANKL and JNK2 were increased while that of Bcl-2 was decreased after treatment with RGD-FasL, suggesting that RGD-FasL induces apoptosis through caspase activation. We concluded that RGD-FasL could possibly be considered as a novel therapeutical candidate for the treatment of pituitary adenomas. *Cellular & Molecular Immunology*. 2008;5(1):61-68.

Key Words: RGD-FasL, pituitary adenoma, apoptosis

Introduction

The genesis of pituitary adenomas is believed to be involved in cell transformation due to genetic mutations, followed by central, peripheral and local signals that induce cell proliferation, thus stimulating tumor progression (1). Considering that the cells bearing genetic alterations are physiologically eliminated by apoptosis, defects in cell death processes play an important role in tumor genesis and growth (2). Clinically, the majority of pituitary adenomas are benign, however, invasiveness or infiltration of adjacent tissues (bone, dura, or cavernous sinus) may serve as an important factor adversely affecting the outcome of tumor and compromising the disease-free survival of patients (3-5). Thus, novel approaches are needed to eliminate pituitary adenomas.

Fas is a type-I membrane protein that has a conserved domain called the death domain. Fas ligand (FasL) is a member of the tumor necrosis factor (TNF) family (6). Fas interacting with FasL results in formation of a death-inducing signaling complex (DISC), which initiates a cascade of events that, through activation of caspases 8, lead to apoptosis (7, 8) The initiator caspases can either directly activate executioner caspase 3 or cleave Bid, a proapoptotic member of the Bcl-2 family. The truncated Bid translocates to the mitochondria stimulating cytochrome c release (9). Together with Apaf-1 activates caspase 9, cytochrome c in turn activates caspase 3 (10). Then, caspase 3 and other downstream caspases cleave subtrates involved in cell disassembling (11). It was reported that FasL-dependent

[1]Department of Neurosurgery, Zhongshan Hospital, Xiamen University, Xiamen, Fujian 361004, China;

[2]Cancer Research Center, Medical College, Xiamen University, Xiamen, Fujian 361000, China;

[3]School of Life Science, Xiamen University, Xiamen, Fujian 361000, China;

[4]Department of Neurosurgery, Xiangya Hospital, Zhongnan University, Changsha, Hunan, China;

[5]Corresponding to: Dr. Lukui Chen, Department of Neurosurgery, Zhongshan Hospital, 201-209, South Hubin Street, Xiamen, Fujian 361004, China. Fax: +86-592-221-2328, E-mail: neuro_clk@hotmail.com

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Abbreviations: FasL, Fas ligand; DcR, decoy receptor; RGD, tripeptide sequence Arg-Gly-Asp; RGD-FasL, fusion protein of RGD and Fas ligand; TNF, tumor necrosis factor; RT-PCR, reverse transcription PCR; mAb, monoclonal antibody; DISC, death-inducing signaling complex; OD, optical density; AI, apoptosis index; ANOVA, analysis of variance; JNK, c-Jun N-terminal kinase; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; CRD, cysteine-rich domains.

apoptosis played a critical role in peripheral deletion, a process that eliminates activated lymphocytes at the end of an immune response; in addition, FasL-mediated apoptosis contributed to elimination of virus-infected cells or cancer cells by cytotoxic lymphocytes (12, 13).

The tripeptide sequence Arg-Gly-Asp (RGD) is a well known motif recognizing and interacting with integrin, a family of transmembrane heterodimeric glycoproteins composed of one α and one β subunits (14). Proteins that contain the RGD attachment site, together with the integrins that serve as receptors for them, constitute a major recognition system for cell adhesion (15). The RGD sequence is the cell attachment site of a large number of adhesive extracellular matrix, blood, and cell surface proteins, and nearly half of the over 20 known integrins recognize and bind to related sequences in their adhesion protein ligands (16). The integrin-binding activity of adhesion proteins can be reproduced by short synthetic peptides containing the RGD sequence. Such peptides promote cell adhesion when insolubilized onto a surface, and inhibit it when presented to cells in solution. As the integrin-mediated cell attachment influences and regulates cell migration, growth, differentiation, and apoptosis, the RGD peptides and mimics can be used to probe integrin functions in various biological systems (17).

Interestingly, Candolfi et al. had reported that Fas activation induces apoptosis of anterior pituitary cells from female rats, suggesting that FasL is involved in the maintenance of tissue homeostasis in the anterior pituitary gland and could have potential benefits for the treatment of pituitary adenomas (18-21). In the present study, we detected the expressions of Fas and DcR3 on 3 pituitary adenoma cell lines. In addition, we investigated the cytotoxic effects on these tumor cells induced by FasL and RGD-FasL (produced by Zhuang), and the underlying mechanism.

Materials and Methods

Pituitary adenoma cell lines

GH3/MMQ/AtT20 cell lines (purchased from the Cell Center, Institute for Basic Medicine, Chinese Academy of Medical Science, Beijing, China) were cultured in MEM containing 10 μl/ml non-essential amino acids, 10 μl/ml L-glutamine, 10 μl/ml penicillin/streptomycin and 10% fetal calf serum 24 h prior to RAd virus infection.

Productions of recombinant FasL and RGD-FasL

The FasL gene was constructed by over-lapping PCR, then was cloned into pGEX-5X-1, which contained a GST tag. RGD was fused onto the N-terminal of FasL protein. The two proteins used in this study were purified through a GST restin column (Novagen, USA).

RT-PCR analysis for expression of Fas/DcR3 mRNA

Total RNA (2 μg) extracted using Trizol Reagent from these cells was reverse-transcribed with 20 U of reverse transcriptase using the Superscript TM II Kit (Gibco-BRL, USA) according to the manufacturer's protocols.

Flow cytometric analysis for Fas/DcR3 expression

All cells (1×10^5/ml) were incubated with anti-Fas mAb and anti-DcR3 mAb (100 μl/ml) (Sigma, USA) in PBS contained 0.5% BSA for 30 min on ice. The cells were washed in cold PBS three times, followed by incubation with a FITC-conjugated affinity-purified goat anti-rabbit IgG (Sigma, USA) for 30 min on ice. After washing, the cells were analyzed on a FACScan (Becton Dickinson, USA).

MTT analysis

The effects of FasL and RGD-FasL on cell proliferation were measured using an MTT based assay. Briefly, the cells (1×10^4/ml) were incubated in triplicate in a 96-well plate (Costar, Cambridge, MA, USA) in the presence of various concentrations of FasL and RGD-FasL (1.875, 3.75, 7.5, 15, 30, 60 μg/ml) in a final volume of 200 μl for the indicated times. Thereafter, 20 μl of MTT solution (5 g/L) was added to each well and then incubated for 12 h. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved in 150 μl of DMSO and then the optical density (OD) value was measured at 490 nm by a multiscanner autoreader (Dynatech MR 5000, Chantilly, VA). The following formula was used: cell proliferation inhibited (%) = [1 - (OD of the experimental samples / OD of the control)] × 100%.

DNA fragmentation analysis

Cells (1×10^6/ml) at exponentially growing phase were treated with or without FasL and RGD-FasL (60 μg/ml) for 4 h. The cells were washed twice with cold PBS, pelleted by centrifugation and lysed in 20 ml lysis buffer (50 mM Tris-HCl, pH 7.4; 10 mM EDTA and 0.5% sodium N-lauroylsarcosinate). Lysate was incubated sequentially with 500 mg/ml ribonuclease A at 50°C for 30 min and 500 mg/ml proteinase K at 50°C for 60 min in a shaking water bath. Equivalent amounts of DNA (2-3 mg) were then analyzed by 2% agarose gel electrophoresis at 50 V in TBE buffer (2 mM EDTA, pH 8; 89 mM Tris-HCl and 89 mM boric acid). The DNA fragmentation pattern was visualized and photographed under transmission UV light after ethidium bromide staining.

Flow cytometric analysis of cell cycle and apoptosis

Cell cycle distribution and apoptosis was determined by staining DNA with propidium iodide (PI). Briefly untreated or FasL/RGD-FasL treated cells (30 μg/ml, 12 h) were centrifuged, washed in PBS and fixed in 70% pre-cooled ethanol. The tubes containing the cell pellets were stored at 4°C for at least 24 h. After this the cells were centrifuged at 1,500 rpm for 5 min, and the supernatant was discarded to remove ethanol completely. The pellets were washed by PBS and stained with PI solution [100 μg/ml PI from 50× stock solution (2.5 mg/ml), 0.1 mg/ml RNase A, 0.05% Triton X-100], incubate in the dark for 40 min at 37°C. After PBS of 3 ml was added, the cells were pellet (1,500 rpm, 5 min) and the supernatant were taken off. Then the pellet was resuspend in 500 μl PBS for flow cytometric analysis. Cells with DNA content below G1 phase (peak of hypodiploid DNA below G1) were regarded as apoptotic cells. The

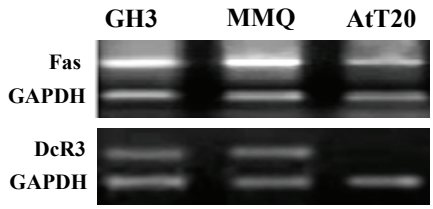

Figure 1. mRNA expressions of Fas/DcR3 were measured by RT-PCR analysis. Total RNA was isolated from each cell line and subjected to RT-PCR analysis. The RT-PCR products were analyzed on 1.5% agarose gel electrophoresis. Fas was located in 467 bp, DcR3 in 320 bp and GAPDH (internal control) in 189 bp.

percentage of cells in the G0/G1 phase and G2/M phase were analyzed by multicycle software.

Western blotting analysis

Aliquots (30 μg of protein) of cell lysates were separated on 12% SDS-PAGE, blotted onto a nylon membrane and probed with antibodies against caspase-8/9/3, RANKL, JNK2 and Bcl-2 (Sigma, USA). Membranes were washed with 0.05% (vol/vol) Tween 20 in PBS (pH 7.6) and incubated with 1 : 2,000 dilution of horseradish peroxidase-conjugated secondary Abs (Promega, USA) for 60 min at room temperature. Protein bands were visualized by DAB reaction.

Caspase inhibition assay

Whether RGD-FasL induce tumor cell apoptosis through caspase pathway was further measured using an MTT based assay. Briefly, the cells (5,000/well) incubated in a 96-well plate (Costar, Cambridge, MA, USA). RGD-FasL (IC_{50}) and various concentrations of caspase inhibitor (5, 10, 15, 20, 25, 30, and 35 μM) were added at the same time in a final volume of 200 μl for the indicated times. Then the inhibition effects were measured by MTT analysis.

Statistical analysis

Data were presented as the mean ± SD per group. Statistical analysis was performed using Student *t*-test and ANOVA followed by multiple comparison tests. A p-value < 0.05 was considered to be statistically significant. Experiments, both the treated group and the control, were carried out in triplicates.

Results

mRNA expressions of Fas/DcR3 in tumor cell lines

The mRNA expressions of Fas in three tumor cell lines were displayed without apparent difference. However, the level of DcR3 mRNA expression in AtT20 cells was lower than that in GH3 and MMQ cells (Figure 1). Fas was located in 467 bp, DcR3 in 320 bp and GAPDH in 189 bp.

Surface expressions of Fas/DcR3 in tumor cell lines

We examined the cell surface expressions of Fas/DcR3 on different cell lines by flow cytometry using the anti-Fas mAb and anti-DcR3 mAb. Approximately 13.7% of GH3 cells,

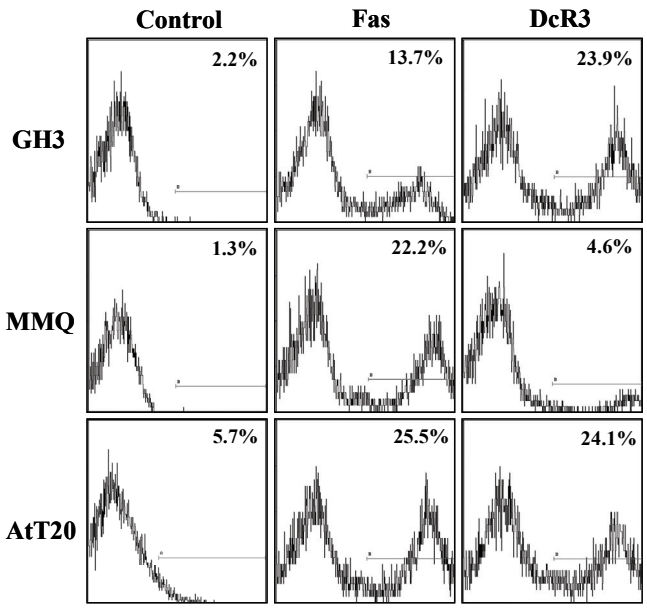

Figure 2. Surface expressions of Fas/DcR3 on cell lines measured by flow cytometry. The cells were incubated with anti-Fas mAb or anti-DcR3 mAb for 30 min on ice. The cells were washed three times, followed by incubation with a FITC-conjugated affinity-purified goat anti-rabbit IgG for 30 min. After washing, the cells were analyzed by FACS.

25.5% of MMQ cells, 22.2% of AtT20 cells expressed Fas, while about 23.9% of GH3 cells, 24.1% of MMQ cells, 4.6% of AtT20 cells expressed DcR3 (Figure 2).

Cytotoxic effects of FasL/RGD-FasL on tumor cell lines

The cytotoxic effects of FasL and RGD-FasL on 3 tumor cell lines were all taken in a dose-dependent manner. However, the sensitivities of tumor cell lines GH3/MMQ/AtT20 to FasL/RGD-FasL were different in groups. Statistically, cell lines MMQ/AtT20 showed the same sensitivity to RGD-FasL as to FasL, while cell line GH3 was less sensitive to RGD-FasL than to FasL. The concentrations of FasL/RGD-FasL needed to inhibit cell growth of GH3 were higher than those for MMQ and AtT20 cells. Their IC_{50} values at 12 h were 53.2 ± 1.1 μg/ml / 56.2 ± 0.8 μg/ml, 34.4 ± 0.5 μg/ml / 33.3 ± 1.4 μg/ml, and 47.3 ± 0.7 μg/ml / 43.3 ± 0.6 μg/ml, respectively (Figure 3).

Morphological change by optical microscope

After treatment with RGD-FasL at 60 μg/ml for 4 h, the cells shrinked, and developed bubble-like blebs on their surface. Apoptotic cells break into smaller pieces called apoptotic bodies that other body cells recognized and ate (Figure 4). It suggests that RGD-FasL could induce three cell apoptosis.

Induction of apoptosis by FasL/RGD-FasL

Genomic DNA fragmentation as a hallmark of apoptotic cell death was confirmed using an agarose gel electrophoresis. After treatment of cells with FasL/RGD-FasL, at 60 μg/ml

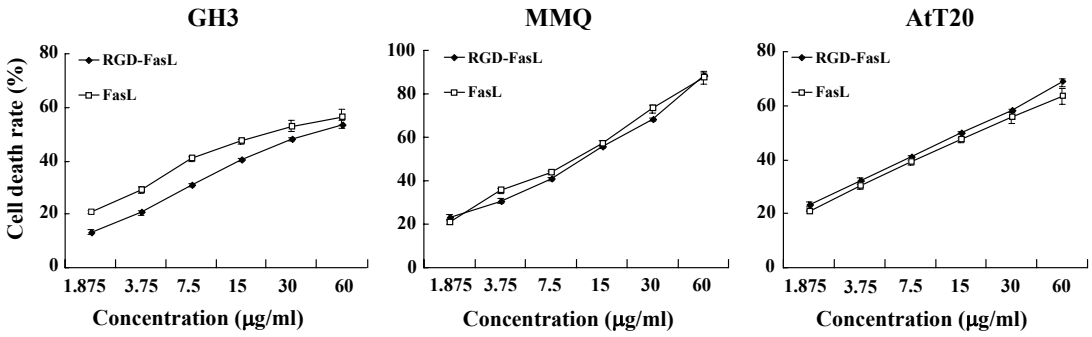

Figure 3. Cytotoxic effects of FasL/RGD-FasL on GH3, MMQ and AtT20 cells. The cells were treated with FasL/RGD-FasL from 1.875 μg/ml to 60 μg/ml for 12 h. The cytotoxic effects of FasL/RGD-FasL on 3 tumor cell lines were all taken in a dose-dependent manner. Cell lines MMQ/AtT20 showed the same sensitivity to RGD-FasL as to FasL ($p > 0.05$), while cell line GH3 was less sensitive to RGD-FasL than to FasL ($p < 0.05$). The concentrations of FasL/RGD-FasL needed to inhibit cell growth of GH3 were higher than those for MMQ and AtT20 cells ($p < 0.05$).

for 4 h, a typical ladder pattern of internucleosomal DNA fragmentation was observed compared with control groups (Figure 5). This indicates that RGD-FasL could induce three cell apoptosis.

FasL/RGD-FasL inducing change of cell cycle and apoptosis in tumor cells

After treatment with FasL/RGD-FasL at the concentration of 30 μg/ml for 12 h, the numbers of G0/G1 and G2/M phase in GH3/AtT20 cells were significantly increased and decreased respectively comparing with the control group; the numbers of both G0/G1 and G2/M phase in MMQ cells were decreased comparing with the control group. In MMQ and AtT20 cells treated with RGD-FasL, the AI was not significantly different from that treated with FasL, while in GH3 cells treated with RGD-FasL, the AI was lower than that treated with FasL.

The analysis of DNA contents showed the numbers of hypodiploid DNA (point to apoptosis) before G1 phase in GH3 cells were 38.8% of those treated with RGD-FasL at the concentration of 30 μg/ml for 12 h, while 55.8% in MMQ cells, and 49.5% in AtT20 cells (Figure 6).

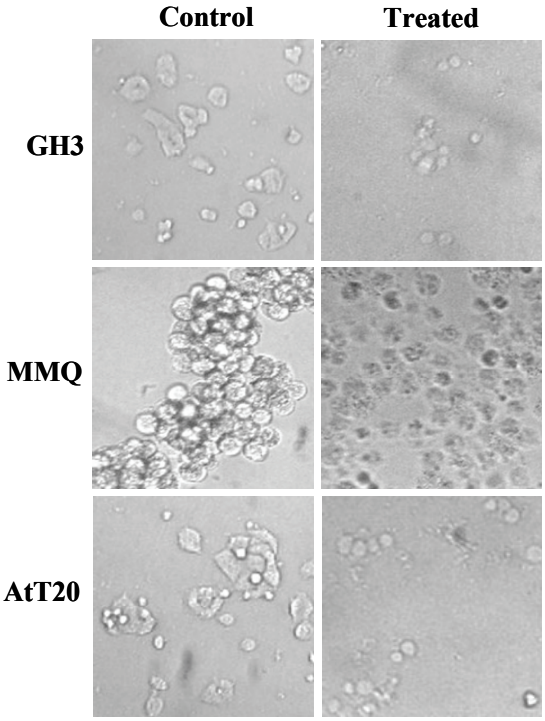

Figure 4. The treated GH3/MMQ/AtT20 cells were observed under optical microscope. Cells were cultured with 60 μg/ml RGD-FasL for 4 h. After that, they shrink, develop bubble-like blebs on their surface, then break into smaller pieces called apoptotic bodies.

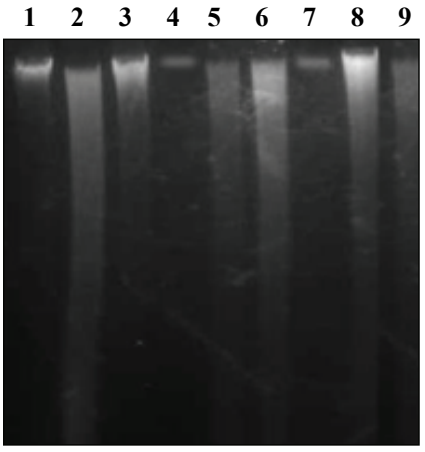

Figure 5. Induction of apoptosis by FasL/RGD-FasL. After treatment of cells with FasL/RGD-FasL at 60 μg/ml for 4 h, a typical ladder pattern of internucleosomal DNA fragmentation was observed comparing with control groups. Lane 1, GH3; Lane 2, GH3 + FasL; Lane 3, GH3 + RGD-FasL; Lane 4, AtT20; Lane 5, AtT20 + FasL; Lane 6, AtT20 + RGD-FasL; Lane 7, MMQ; Lane 5, MMQ + FasL; Lane 6, MMQ + RGD-FasL.

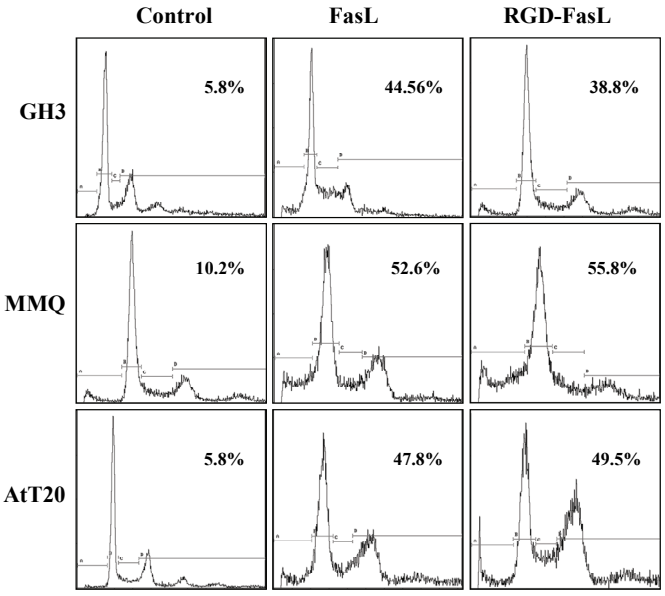

Figure 6. FasL/RGD-FasL inhibiting GH3/MMQ/AtT20 cell growth and inducing cell apoptosis. All cells were cultured with 30 μg/ml RGD-FasL/FasL for 12 h.

Western blotting detection of caspase-8/9/3, Bcl-2, RANKL, JNK2

Analysis of the expression of the proteins involved in the apoptosis pathway revealed that after giving FasL/RGD-FasL at the concentration of 60 μg/ml for 12 h, the expression of caspase-8/9/3 in GH3/MMQ/AtT20 cells were up-regulated compared with control groups and its 31 kDa proteolytic fragments were detected (data not shown). It was noted that the expression of caspase-8/9/3 in GH3 cells treated with FasL was higher than that treated with RGD-FasL. After giving FasL/RGD-FasL under the same conditions, the expression of Bcl-2 in three cells was decreased obviously. It indicated that RGD-FasL inducing the apoptosis of three tumor cells should be through caspase pathway. Next, we further studied other factors involved in this treatment. The expression of RANKL and JNK2 in GH3/MMQ/AtT20 cells were up-regulated compared with control groups. Cell lines MMQ and AtT20 showed obvious RANKL and JNK2 expressions, while in GH3 cells the expressions of RANKL and JNK were lower (Figure 7), suggesting RGD-FasL inducing apoptosis may activate the JNK pathway to stimulate the expression of receptors.

In order to prove the mechanism of RGD-FasL inducing apoptosis *via* caspase pathway, we added various concentrations of caspase inhibitor to GH3, MMQ, AtT20 cells treated with RGD-FasL. The results showed that caspase inhibitor could inhibit the apoptosis induced by RGD-FasL. The inhibition effect has presented dose dependence and reached saturation when the concentration of inhibitor increased, suggesting RGD-FasL inducing tumor cells apoptosis must be through caspase pathway (Figure 8) .

Discussion

Targeted delivery enhances chemotherapeutic effect and spares normal tissues from the toxic side effects of powerful drugs. Antiangiogenic therapy, for example, prevents neovascularization by inhibiting proliferation, migration and differentiation of endothelial cells (22). Furthermore, chemotherapy that induces vessel wall or endothelial cell death results in loss of vascular integrity, platelet aggregation and vascular occlusion, denying oxygen delivery and precipitating tumor cell death downstream (23). The identification of molecular markers that differentiate newly formed capillaries from their mature counterparts (24, 25) has paved the way for targeted delivery of cytotoxic agents to the tumor vasculature (26). The $\alpha_V\beta_3$ integrin is one of the most specific of these unique markers. It is only during angiogenesis can this marker be "seen" by targeting agents that are restricted to the vascular space (27-29). Encouragingly, the high affinity $\alpha_V\beta_3$ selective ligands, RGD have been identified by phage display studies (30). In our study, the RGD peptide used for newly-developed RGD-FasL has a conformationally restrained RGD sequence that binds specifically and with high affinity to $\alpha_V\beta_3$. The RGD-FasL produced by Zhuang was designed

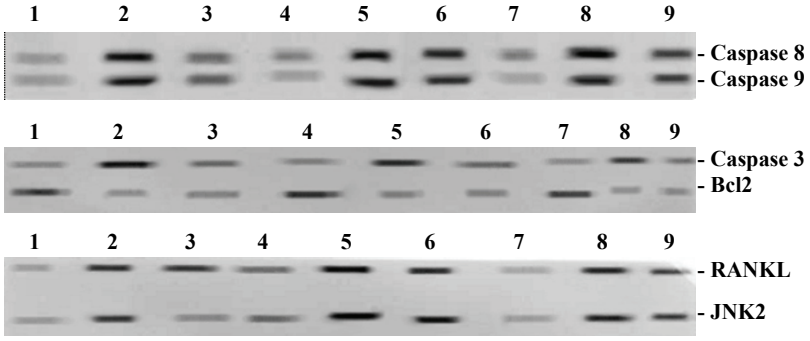

Figure 7. Detection of levels of caspase-8/9/3, Bcl-2, RANKL, JNK2 protein expression in 3 cell lines by Western blot analysis. Lane 1, GH3; Lane 2, GH3 + FasL; Lane 3, GH3 + RGD-FasL; Lane 4, MMQ; Lane 5, MMQ + FasL; Lane 6, MMQ + RGD-FasL; Lane 7, AtT20; Lane 8, AtT20 + FasL; Lane 9, AtT20 + RGD-FasL.

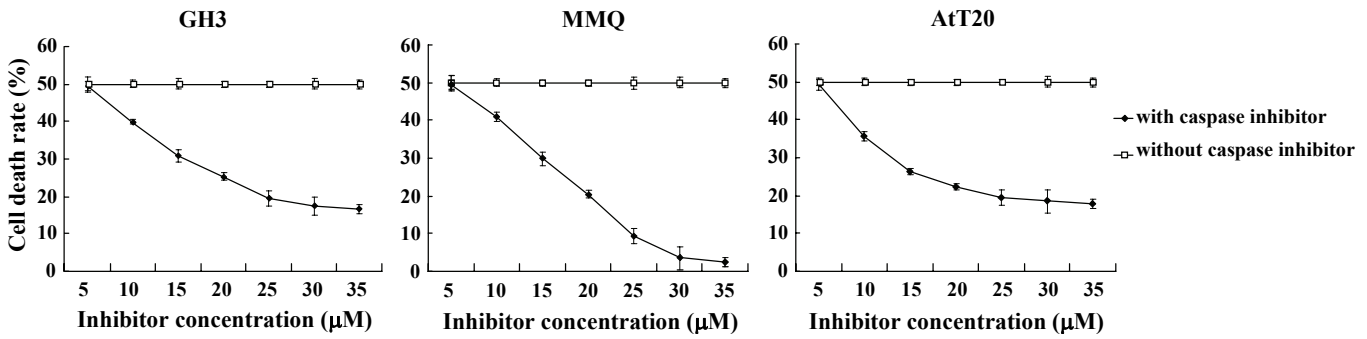

Figure 8. Caspase inhibition assay. The cells treated with RGD-FasL (IC_{50}) were added with various concentrations of caspase inhibitor from 5 μM to 35 μM for 12 h. The inhibitory effects of RGD-FasL on 3 tumor cell lines were all taken in a dose-dependent manner.

to carry an $\alpha_V\beta_3$ selective ligand, allow active targeting and delivery of effector substances to sites of tumor angiogenesis. There have been a series of inspiring tests showing that the RGD-FasL exerts cytotoxic activity more than FasL alone does on U138 and Hela cell lines (data not shown).

Jaita et al. detected that Fas and FasL are expressed in several anterior pituitary cell types from female rats, mainly in lactotropes and somatotropes. In addition, they reported that Fas activation induces apoptosis of lactotropes and somatotropes as well (21). It was also demonstrated that the somatolactotrophic tumor GH3 cell line and the corticotrophic tumor AtT20 cell line express Fas (31, 32). In GH3 and AtT20 cells, overexpression of Fas strongly enhanced cell death by apoptosis, suggesting that death receptor-apoptotic pathways are conserved in both tumoral cell lines. Considering that apoptosis plays a central role in regulation of pituitary tissue homeostasis, the imbalance between cell death and proliferation in favor of cell survival could result in tumor formation. One of the commonly employed strategies in experimental gene therapy for cancer is to target death receptors specifically to trigger apoptosis in tumor cells (33-36).

DcR3 has four cysteine-rich domains (CRDs) and is a secreted, soluble protein. It binds to FasL with an affinity which equals that of Fas and competes with Fas for FasL-binding; further, it inhibits apoptosis induced by FasL, which suggests that it is a decoy receptor for FasL. Studies with primary lung and colon tumors show substantial amplification of the DcR3 gene in about half of the tumors examined and frequent DcR3 mRNA overexpression in malignant tissue. Given that FasL is an important mediator of tumor cell killing by natural killer cells and cytotoxic T cells, the genomic amplification of DcR3 suggests that certain tumors may escape immune cytotoxic attack by means of DcR3 overexpression. Thus therapeutic strategies could be devised to inhibit the interaction of DcR3 with FasL and potentially to enhance the antitumor immune response of cancer patients whose tumors overexpress DcR3 (37).

In our present study, there was not apparent difference of Fas mRNA expressions in 3 pituitary adenoma cell lines, while the level of DcR3 mRNA expression in AtT20 cells was lower than that in GH3 and MMQ cells. The results were consistent with those examined by flow cytometry. Whether the existence of DcR3 on tumor cells or the difference of mRNA expressions between Fas and DcR3 presented here could be the cause of selective apoptosis induced by FasL remains unclear and needs to be further determined.

In addition, we examined the antiproliferative activity of RGD-FasL against three tumor cell lines. Evidently, their antiproliferative effects were gradually augmented with the increased concentration (dose-dependent manner). These results suggested that the activity may be due to the direct toxicity against tumor cells. Interestingly, we observed that the sensitivities of cell lines GH3, MMQ and AtT20 to FasL/RGD-FasL were statistically different in groups. Cell line MMQ and AtT20 showed the same sensitivity to RGD-FasL as to FasL, while cell line GH3 was less sensitive to RGD-FasL than to FasL. We suspect that the structure and different binding activities of RGD to its receptors (integrins) on tumor cells could probably change the activity of FasL, thus influence the cytotoxic effects of the RGD-FasL complex. The results, however, demonstrated that RGD-FasL basically induced apoptosis of 3 tumor cell lines and remained functional as FasL did. Since RGD alone has been confirmed to specifically attach the tumor vasculature, and inhibit the angiogenesis induced by tumor tissues (17), it hence reduced the dosage intravenously. Moreover, compared to FasL, RGD-FasL has no obvious effect on induing cell apoptosis; it might be relevant to the expression of integrins on three cells (data not shown).

Next, whether the apoptotic pathway is involved in the cell death caused by RGD-FasL was investigated. Several lines of evidence are presented in our study for the first indication that apoptosis is the mode of cell death caused by this complex. Morphological characterizations were clearly observed after 4 h. Consistent with this observation, the nuclear fragments resulting in a ladder formation was also evident. Cleavage of DNA at the internucleosomal linker sites yielding DNA fragments in multiples of 180 base pairs is regarded as a biochemical hallmark of apoptosis (38). The result of cell cycle and apoptosis analysis assessed by flow cytometry with PI staining, indicated that the apoptosis rate in three cell lines is increased after treatement with RGD-FasL or FasL. Also, the result strongly suggested that

RGD-FasL could inhibit cells in G0/G1 phase and G2/M phase, hence inhibit cell growth. In MMQ and AtT20 cells treated with RGD-FasL, the AI was not significantly different from that treated with FasL, while in GH3 cells treated with RGD-FasL, the AI was lower than that treated with FasL. The data summarized in the optimal experimental condition assessed by flow cytometry are consistent with the result of MTT.

It is clear that caspase family members play important roles in driving apoptosis. Activation of caspase appears to be directly responsible for many of the molecular and structural changes in apoptosis. Caspase-8 is the prototypical apoptosis initiator caspase downstream of TNF super-family death receptors. With substrates that include apoptosis-related effector caspases and pro-apoptotic Bcl-2 family members, active caspase-8 is capable of unleashing cascades of cellular events that can result in apoptosis (39). Indeed, caspase-8-deficient cells are resistant to death receptor-mediated cell death both *in vitro* and *in vivo*. Our observation revealed that caspase-8/9/3 could be activated by RGD-FasL in a dose-dependent manner, suggesting that RGD-FasL induced apoptosis in three tumor cell lines through caspase pathway. And the caspase inhibition assay also further supported this viewpoint. After giving FasL/RGD-FasL under the same conditions, the expression of Bcl-2 in three cells was decreased obviously. It revealed that the expression of Bcl-2 had an important effect on anti-apoptosis of tumor cell lines. Our results also showed that the expression of RANKL and JNK2 in GH3, MMQ and AtT20 cells were consistent with the expression of caspase-8/9/3, indicating RGD-FasL passed the apoptosis signal through caspase activation, then increased the expression of RANKL and JNK2, affecting the DNA replification, maybe activated the expression of receptors, then further stimulated cell apoptosis. In our opinion, the sensitivities of RGD-FasL could be relevant to the mRNA expressions of their receptors and the release of caspase-8/9/3, RANKL, JNK2 and Bcl-2.

In conclusion, our results demonstrate that functionally, RGD-FasL has nearly the same effective activity as FasL dose on pituitary adenoma cells. Our study *in vitro* for the first time suggests that RGD-FasL could probably be considered as a novel therapeutical candidate for the treatment of pituitary adenomas. Further studies are currently undergoing on animal models to determine the selective cytotoxicity of RGD-FasL towards tumor-associated vasculature and tumor cells.

Acknowledgements

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