Article

Correlation between Expression of DcR3 on Tumor Cells and Sensitivity to FasL

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To investigate the correlation between sensitivity to Fas ligand (FasL) and expression level of decoy receptor 3 (DcR3) on tumor cell surface, Fas/DcR3 mRNA expression was detected by RT-PCR. Anti-DcR3 mAb was used to detect expression level of DcR3 on surface of tumor cells by flow cytometry. Caspase-8, caspase-9, caspase-3, Bcl-2 expressions were analyzed by Western blot, respectively. Sensitivity to apoptosis induced by FasL was determined by Annexin V apoptosis kit. The expressions of DcR3 on the surface of tumor cells from high to low were approximately 35.3% in BGC823 cells, and 21.6% in MCF-7 cells, respectively. The apoptotic rates induced by FasL from low to high were 15.6% in BGC823 cells, and 58.2% in MCF-7 cells, respectively. There was a significant correlation between the expression levels of DcR3 with FasL-inducing apoptosis. *Cellular & Molecular Immunology*. 2007;4(6):455-460.

Key Words: DcR3, FasL, Fas, apoptosis

Introduction

Fas ligand (FasL), a type II membrane protein, belongs to the tumor necrosis factor (TNF) superfamily (1). FasL is expressed by activated T lymphocytes and natural killer (NK) cells, and by a small number of non-lymphoid cells (2). The expression of FasL has also been reported in non-immune cells, mainly from immune-privileged tissues such as testis, cornea, trophoblast, and cancer cells, which suggests that the Fas-FasL system may play an important role in the privileged status (3-5). The Fas (CD95) antigen, a 45-kDa protein of the TNF receptor (TNFR) family, is widely expressed and binds FasL (6). By engaging its receptor (Fas) membrane-bound FasL induces apoptosis in the target cell and, in this way, FasL plays a central role in both cell-mediated immunity and immune down-regulation (7). Hiroshi Arai, et al. reported that transfection of FasL gene into the CT26 colon carcinoma which did not express Fas inhibited its growth *in vivo*. These findings suggest that gene transfer of FasL generates apoptotic responses (8).

Recent studies have suggested that resistance to apoptosis with loss of Fas function may play an important role in the pathogenesis of several malignancies (9). In addition, Decoy receptor 3 (DcR3) was recently reported, which is a decoy receptor for FasL. DcR3 lacks an apparent transmembrane sequence, may be a secreted, rather than a membrane-associated receptor. Pitti et al. (10) found that the DcR3 gene was amplified in approximately half of various human lung tumors and adenocarcinomas and suggested that over-expression of DcR3 receptor may exist in these tumors, meaning that the growth advantage of tumor cells was due to blockade of FasL-induced cell death (11).

It has been reported that there are two kinds of FasL receptors, death receptors and decoy receptors. Fas can transfer death signal, resulting in cell apoptosis (12). DcR3 doesn't have death domain, so it couldn't transfer death signal (13). In this study, we examined the effect of DcR3 expression on tumor cell lines, finding the correlation between sensitivity to FasL inducing apoptosis in tumor cell lines and the expression of DcR3.

Materials and Methods

Cell culture

Hela (cervical carcinoma cell), BGC823 (gastric carcinoma

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Abbreviations: TNF, tumor necrosis factor; DcR3, decoy receptor 3; FasL, Fas ligand; NK, natural killer; IPTG, isopropyl-β-D-Thiogalactoside; LB, Luria-Bertani; PI, propidium iodide.

cell), MGC803 (gastric carcinoma cell), MCF-7 (breast carcinoma cell), BEL7402 (hepatocellular carcinoma cell), H9101 (hepatocellular carcinoma cell), and HL60 (human promyelocytic leukemia cells) were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and 2 mM glutamine.

Expression and purification of recombinant human FasL in Escherichia coli

The plasmid pET-22b(+)-FasL for the expression of rhFasL was constructed previously by over-lapping PCR in our laboratory. Plasmid DNA which contains a six-histidine tag was then transformed into the *E. coli* Rosetta-gami (Invitrogen). Further purification was carried out through a nickel ion column (Novagen, USA).

Analysis DcR3 expression of tumor cell lines by flow cytometry

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

RT-PCR analysis for expression of Fas/DcR3 mRNA

All cells treated with or without rhFasL (1×10^7/ml) were harvested by centrifugation. Total RNA (2 μg) was extracted using Trizol reagent from these cells, then RNA was reverse-transcribed with 20 U of reverse transcriptase using the Superscript TM II Kit (Gibco-BRL, USA) according to the manufacturer's instructions.

MTT analysis

The effect of rhFasL on cell proliferation was measured using an MTT (Microtiter-tetrazolium, Sigma, USA) based assay. Briefly, the cells (5,000/ml) were incubated in triplicate in a 96-well plate (Costar, Cambridge, MA, USA) in the presence of various concentrations of rhFasL (1.5625, 3.125, 6.25, 12.5, 25, 50 μg/ml) in a final volume of 0.2 ml 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 0.15 ml of DMSO and then the optical density (OD) value was measured at 490 nm by a multiscanner autoreader (Dynatech MR 5000, Chantilly, VA, USA). The following formula was used: cell proliferation inhibited (%) = [1 - (OD of the experimental samples / OD of the control)] × 100%.

Apoptosis assay by flow cytometry

To determine the apoptosis, rhFasL treated or not treated cells (25 μg/ml, 1 h) were washed in PBS and resuspended in binding buffer at a concentration of 1×10^6 cells/L. After incubation, 195 μl of the solution was transferred to a 5 ml culture tube with 5 μl Annexin V-FITC (BD, USA) added. The tube was then incubated for 30 min at room temperature in the dark. The cells were washed with binding buffer and resuspended in 190 μl binding buffer, with 10 μl PI added. Finally, the tube was gently vortexed and incubated for another 30 min in the dark. Cells were analyzed by FACSCalibur with CellQuest software.

DNA fragmentation

DNA fragmentation served as a late apoptosis marker was detected by agarose gel electrophoresis. Cells (1×10^6 cells/ml) at exponentially growing phase were treated with or without rhFasL (25 μg/ml) for 4 h. The cells were washed twice with cold PBS, pelleted by centrifugation and lysed in 2 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.

Western blot

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, Caspase-3, Caspase-9 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 peroxides-conjugated secondary Abs (Promega, USA) for 60 min at room temperature. Protein bands were visualized by DAB (3, 3'-Diamino-benzidine) reaction.

Statistical analysis

Data were presented as the mean ± SD per group. Statistical analysis was made for multiple comparisons using analysis of variance and Student *t* test. The p value < 0.05 was considered to be statistically significant.

Results

The rhFasL expression and purification

The *E. coli* expression system was used to prepare rhFasL in inclusion form. After isolated and purified, the purity of the target protein was above 98%. On SDS-PAGE, the mobility of the purified protein was found to correspond to a molecular weight of 35 kDa (Figure 1). The purified protein was further examined by Western blot using anti-human FasL antibody. As shown in Figure 1, the rhFasL migrated at 35 kDa as expected and no degradation was observed.

Surface expression of DcR3 on different cell lines

We examined the cell-surface expression of DcR3 on different cell lines by flow cytometry using the anti-DcR3 mAb. As represented in Figure 2, approximately 35.3% of BGC823 cells, 31.9% of Hela cells, 28.8% of MGC803 cells, 27.5% of BEL7402 cells, 24.9% of H9101 cells, 23.9% of HL60 cells, and 21.6% of MCF-7 cells expressed DcR3.

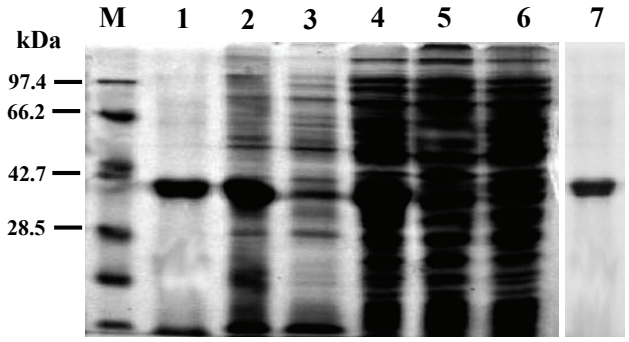

Figure 1. Recombinant rhFasL protein purification and identification. Purified rhFasL proteins were analyzed by SDS-PAGE. Lane M, molecular weight markers; Lane 1, proteins were purified using a Nickel-affinity chromatography column; Lane 2, total insoluble protein; Lane 3, total soluble protein; Lane 4, proteins of colony induced with 0.3 mM IPTG; Lane 5, proteins of colony induced without IPTG; Lane 6, proteins of colony with plasmid pET-22b(+) induced with 0.5 mM IPTG; Lane 7, Western blot analysis of rhFasL protein using anti-human FasL antibody raised against goat, HRP-conjugated anti-goat antibody was used as secondary antibody.

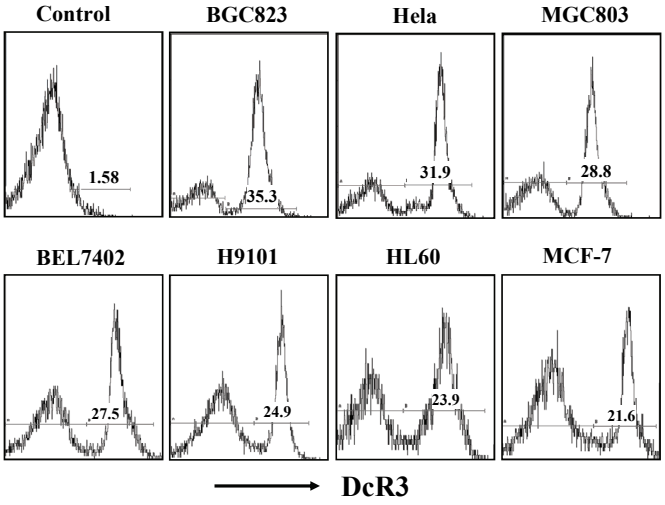

Figure 2. Surface expression of DcR3 on tumor cell lines. The cells were incubated with anti-DcR3 mAb, and stained with FITC-conjugated goat anti-rabbit IgG. Then the cells were analyzed by FACS.

mRNA expression of Fas, DcR3 in tumor cell lines

FasL induces apoptosis in tumor cell lines through its receptors. The levels of Fas and DcR3 mRNA expression in seven tumor cell lines were different. The expression of Fas mRNA exists in all cells with low levels before rhFasL treatment. After giving rhFasL at the concentration of 25 μg/ml for 4 h, the levels of Fas mRNA expression in Hela, MGC803 and BEL7402 were decreased, while the expression of DcR3 mRNA increased. Under the same condition, the levels of Fas and DcR3 mRNA expression in MCF-7, H9101 and HL60 were increased, while the extent of increase expression of DcR3 mRNA was lower than Fas mRNA (Figure 3).

Correlation between apoptosis induced by rhFasL and expression level of DcR3 on tumor cell lines

In order to study the relationship of rhFasL inducing tumor cell apoptosis and DcR3 expression, we firstly used MTT assay to determine the cell growth blocked by rhFasL. The sensitivities of tumor cell lines to rhFasL were different (Figure 4). Cell lines HL60, MCF-7, MGC803, BGC823 and H9101 were sensitive to rhFasL. Cell lines BEL7402 and Hela were partially sensitive to rhFasL in a dose dependent manner. Their IC50 values at 12 h were 12.3 ± 0.7 μg/ml of MGC803, 54.1 ± 1.1 μg/ml of BEL7402, and 6.4 ± 0.5 μg/ml of MCF-7, 22.3 ± 0.7 μg/ml of HL60, 6.1 ± 1.1 μg/ml of BGC823, and 26.4 ± 0.5 μg/ml of H9101, 64.1 ± 1.3 μg/ml of Hela.

Genomic DNA fragmentation as a hallmark of apoptotic cell death was confirmed using an agarose gel electrophoresis. After treatment of cells with rhFasL, at 25 μg/ml for 4 h, a typical ladder pattern of internucleosomal DNA fragmentation was observed comparing with control groups (Figure 5).

We further analyzed the cell death mode induced by rhFasL by flow cytometry. As shown in Figure 6, the HL60,

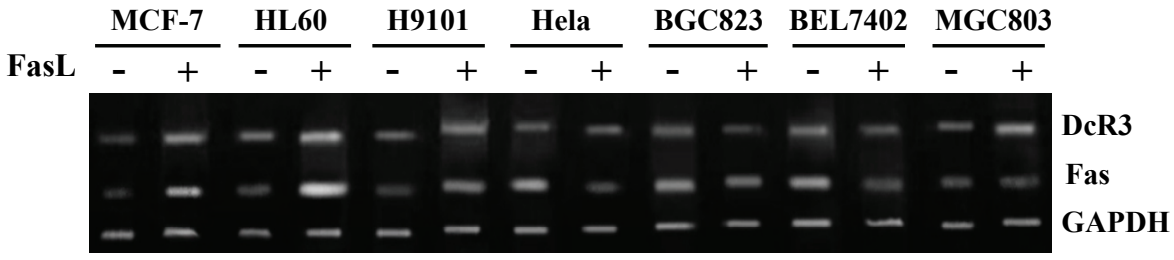

Figure 3. mRNA levels of Fas and DcR3 were measured by RT-PCR. Total RNA was isolated from each sample and subjected to RT-PCR. The RT-PCR products were analyzed on 1.5% agarose gel electrophoresis. The results showed after rhFasL treatment, the level of Fas mRNA expression in Hela, MGC803 and BEL7402 were lower compared with control group, while the expression of DcR3 mRNA increased. With the same condition, the levels of Fas and DcR3 mRNA expression in MCF-7, H9101 and HL60 were increased.

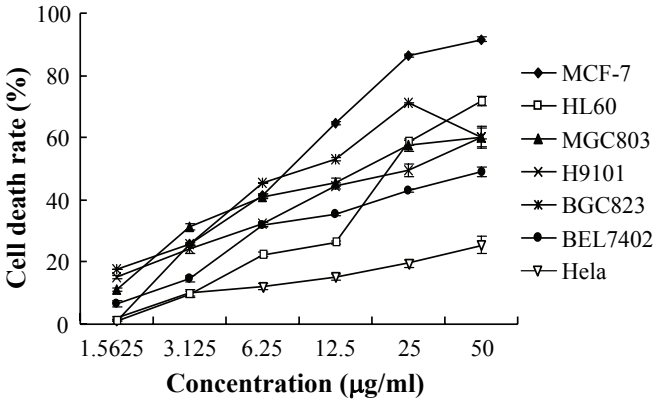

Figure 4. Cytotoxic effects of rhFasL on tumor cell lines. Tumor cell lines (HL60, MCF-7, BGC823, MGC803, BEL7402, Hela and H9101) were treated with rhFasL for 12 h at the indicated concentrations. The results showed that cell lines HL60, MCF-7, MGC803, BGC823 and H9101 were sensitive to rhFasL. Cell lines BEL7402 and Hela were partially sensitive to rhFasL in a dose-dependent manner.

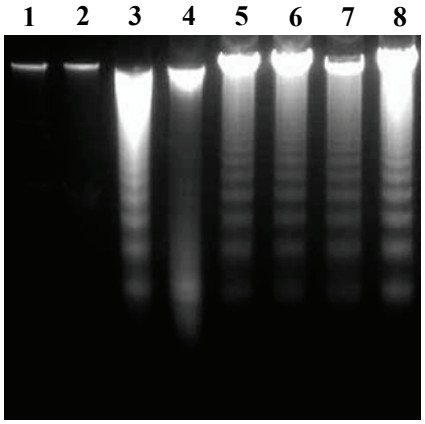

Figure 5. DNA fragmentation was observed undergoing rhFasL-induced apoptosis by agarose gel electrophoresis. Cells treated with 25 μg/ml rhFasL for 4 h. Lane 1, tumor cells without any treatment; Lane 2, Hela treated with rhFasL; Lane 3, MCF-7 treated with rhFasL; Lane 4, MGC803 treated with rhFasL; Lane 5, BGC823 treated with rhFasL; Lane 6, BEL7402 treated with rhFasL; Lane 7, H9101 treated with rhFasL; Lane 8, HL60 treated with rhFasL.

H9101 and MCF-7 cell death mode mediated by FasL were mostly apoptosis, and BEL7402, MGC803 cells death mode mediated by FasL is mostly necrosis, while BGC823 and Hela cells were the secondary necrotic cells.

Analysis of the expression of the proteins involved in the apoptosis pathway revealed that the expressions of Caspase-8, Caspase-3 and Caspase-9, Bcl-2 exist in all cell lines without rhFasL treatment. After treatment with rhFasL at 25 μg/ml for 4 h, the expressions of Caspase-8, Caspase-3 and Caspase-9 in HL60, H9101 and MCF-7 cells were up-regulated compared with control group and their proteolytic fragments were detected (data not shown). While the expression of Bcl-2 in the same cells decreased obviously. Before and after giving rhFasL, the expressions of Caspase-8, Caspase-3 and Caspase-9, Bcl-2 in other tumor cell lines were not distinctly different (Figure 7).

At last, we used statistical methods to study the correlation between apoptosis induced by FasL and expression levels of DcR3 on the surface of tumor cells. The results suggested that the expression levels of DcR3 on surface of tumor cells were relevant to the sensitivity to FasL ($r = 0.9935$, $p < 0.001$, Figure 8).

Discussion

The FasL-Fas receptor interaction is a key physiological

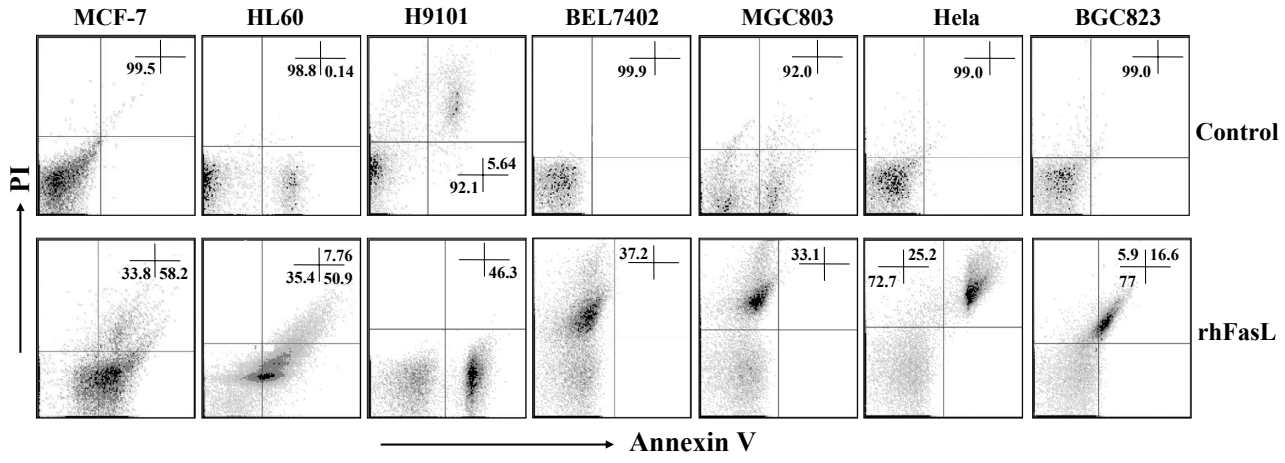

Figure 6. Cell death mode analysis of tumor cells treated with or without rhFasL by flow cytometry. Tumor cells were treated with or without rhFasL (25 μg/ml) for 1 h, and stained with Annexin V and PI. Then the cells were analyzed by FACS.

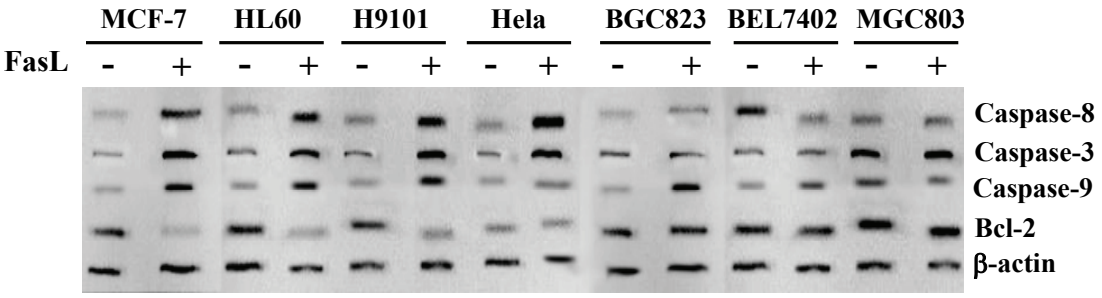

Figure 7. Protein level detection of Caspase-8, Caspase-3, Caspase-9 and Bcl-2 in tumor cell lines by Western blot analysis. Aliquots of cell lysates were separated on SDS-PAGE, blotted onto a nylon membrane and probed with antibodies against Caspase-8, Caspase-3, Caspase-9 and Bcl-2. The HRP-conjugated anti-goat antibody was used as secondary antibody. The results showed that the expression of Caspase-8, Caspase-3 and Caspase-9 in HL60, H9101 and MCF-7 cells were up-regulated compared with control group after rhFasL treatment.

regulator of programmed cell death (14). Stimulation of the Fas receptor by FasL or agonist antibody results in the rapid induction of apoptosis which is dependent upon a signal transduction cascade involving the caspase family of proteases and is independent of new RNA or protein synthesis (15, 16). Apoptosis induced by the Fas receptor is characterized by cell shrinkage, plasma membrane blebbing, and DNA fragmentation into nucleosomal sized fragments.

$Fas^-/FasL^+$ and FasL-$mRNA^+$ HCC cells were detected in the specimen of moderately-differentiated cells (17), suggested that Fas/FasL expression was decreased in proportion to the malignancy of tumor cells. Furthermore, the apoptosis in HCC could be regulated by the suppression of Fas/FasL expression, or sometimes, by the enhancement of FasL expression.

Our studies demonstrated that the apoptosis of tumor cell lines were relevant to DcR3 and Fas mRNA expression. The different mRNA expressions of DcR3 and Fas may be the key point of selectively induction apoptosis of FasL (18, 19).

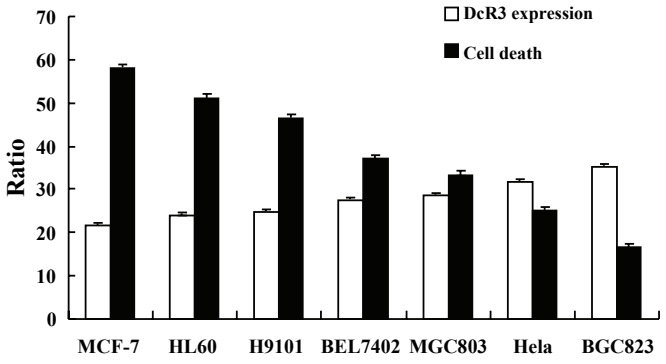

Figure 8. Analysis of the correlation between apoptosis induced by FasL and expression levels of DcR3 on surface of HL60, MCF-7, BGC823, MGC803, BEL7402, Hela, H9101 cells. For calculating cell apoptosis rate, the cells were treated with FasL for 1 h at 25 μg/ml, and then were measured by flow cytometry after staining by Annexin V/PI. For calculating DcR3 expression rate, the cells added with anti-DcR3 mAb, and detected by flow colometry after staining by FITC.

We examined the anti-proliferation activity of rhFasL against tumor cell lines. Interestingly, their anti-proliferation effect was gradually augmented with the increased rhFasL concentration. When the cell lines which have higher DcR3 expression, the anti-proliferation activity of rhFasL was lower. These results suggested that the activity may be due to the DcR3 expression on tumor cells.

DNA fragmentation occurs in cell apoptosis induced by a variety of agents. This cleavage produces ladders of DNA fragments in the size of integer multiples of a nuclesome length (180-200 bp) (20). Due to their characteristic patterns revealed by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. A characteristic ultra-structural appearance of apoptotic cells is observed under an electron microscope (data not shown).

Information summarized above is consistent with the results of cell death mode assessed by Annexin V staining. When the cell lines had higher DcR3 expression, their apoptotic rate induced by rhFasL was lower.

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 (21). In this study, increased expression of Caspase-3 was observed directly in HL60, H9101 and MCF-7 cells after treatment with rhFasL. Distinct activation of Caspase-3, demonstrated by the appearance of proteolytic fragment, was detected 4 hours after FasL treatment. Over-expression of Caspase-8 and Caspase-9 resulting in apoptosis has been reported. The data presented here showed that Caspase-8 and Caspase-9 were up-regulated and cleavage of Caspase-8 was detected in HL60, H9101 and MCF-7 cells (data not shown). Our observations suggested that rhFasL induced apoptosis in tumor cell lines through up-regulation of Caspase-8, Caspase-9 and Caspase-3 activation. Our additional experiments showed that rhFasL could down-regulate the expression of Bcl-2 protein in HL60, H9101 and MCF-7 cells. These results showed that when the

cell lines have higher DcR3 expression, their Caspase-8, Caspase-9 and Caspase-3 expressions were lower and Bcl-2 expressions were higher after rhFasL treatment.

In conclusion, our studies suggested that the sensitivity of tumor cells to rhFasL should be relevant to the DcR3 expression, and the mRNA expressions of its receptors and the release of Caspase-8, Caspase-9, Caspase-3, and Bcl-2. Further studies of its molecular mechanism involved are currently undergoing.

Acknowledgement

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