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ARTICLE

Magnetic nanoparticle-mediated gene therapy to induce Fas apoptosis pathway in breast cancer

Harun Basoglu[1] · Beyza Goncu[2] · Fahri Akbas[3]

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Abstract

CD95 (Fas) is a complex integral protein that can be expressed in many cells. It induces apoptosis when interacted with its ligand CD95L (FasL). However, cancer cells are resistant to CD95-induced apoptosis because of the changes in death domain (DD) of CD95 (procaspase-8 and c-Flip). In this study, magnetic nanoparticles and lipid-based gene transfection methods were performed to provide active Fas expression in breast cancer cells. Plasmid DNA (pDNA), which can express both human Fas and GFP, was transfected to MCF-7 breast cancer cells. Expression of c-FLIP and caspase-8 and effect of monoclonal antibody FasL for apoptosis stimulation were investigated. Also transfection success of methods and effects on surface protein were compared. Western blot results indicated that MCF-7 cells do not express caspase-8 but express large amount of c-FLIP$_L$. Both lipid-based and magnetic nanoparticle-mediated gene transfection methods successfully applied. Caspase-8 apoptosis pathway was activated on transfected cells. Magnetic nanoparticle-mediated gene transfer is a successful non-viral method for transfection, and it does not affect the expression of other cell proteins, such as beta actin and lamin-B1. The raised c-FLIP$_L$ concentration in cytosol inhibits apoptosis. However, transfection of CD95-GFP-tagged pDNA significantly increases apoptosis by activating caspase-8 pathway. FasL interaction indicated a slight increase of apoptosis in the transfected cells. The method and pDNA applied in this study have potentials to be used in gene therapy for breast cancer.

Introduction

CD95 also known as Fas is a member of the death receptor family that can be expressed in many cells but mainly expressed in T-lymphocytes and natural killer cells [1]. CD95 (Fas) is a complex surface protein that induces apoptosis when interacted with its ligand CD95L (FasL) [2]. Binding of CD95L to CD95 causes formation of death-induced signaling complex (DISC), which consist of adopter protein Fas-associated death domain (FADD), procaspase-8, and caspase-8 (FLICE)-like inhibitory protein (c-FLIP) [3, 4]. Both c-FLIP and procaspase-8 connected to FADD via death effector domain. DISC include several isoforms of procaspase-8 (procaspase-8a (p55) and -8b (p53)) and c-FLIP, long isoform c-FLIP$_L$ (55 kDa), short isoform c-FLIP$_S$ (26 kDa), and raji c-FLIP$_R$ (23 kDa) [5, 6] (Supp.Figure-1). Procaspase-8 is essentially responsible of apoptosis pathway; however, c-FLIP$_L$ inhibits the activation of caspase-8. If the amount of c-FLIP$_L$ increases in cytosol when FasL interacted with Fas, apoptosis may not be seen [3, 5, 7]. On the other hand, it is reported that c-FLIP$_L$ functions as an activator for CD95 mediated apoptosis via enhancing procaspase-8 activation [8]. Procaspase -8a and -8b exist in the DISC structure of variety of cells [9]; however, c-FLIP$_L$ concentration may reduce procaspase-8 level while inhibiting interactions of caspase-8 on DISC especially in cancer [10].

Many cancer cells express high level of CD95, whereas they are resistant to CD95-induced apoptosis and multiple mechanisms may play role for the resistance. Cancer cells

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✉ Harun Basoglu
hbasoglu@bezmialem.edu.tr

[1] Department of Biophysics, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey

[2] Department of Biotechnology, Institute of Health Science, Bezmialem Vakif University, Istanbul, Turkey

[3] Department of Medical Biology, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey

can express CD95 and CD95L and it is reported that expression of CD95 and CD95L promotes tumor growth and metastases [10–12]. In addition, elimination of CD95 or CD95L results in cancer cell death independent of caspase-8 [13]. The major reason of Fas apoptosis resistance is activation of nuclear factor (NF)-κB after FasL stimulation, which is related with cFLIP activation [1, 3, 5, 14].

Breast cancer indicates series of genetic mutations and gene therapy could be the solution. The purpose of gene therapy is to deliver a nucleic acid to overcome the cancer proliferation and finally killing the cancer cells. Although clinical trials of gene therapy for cancer is >63%, there are still no approved gene therapy products for breast cancer [15]. Successful nucleic acid transfer is another issue for researchers, and as a non-viral transfection method, magnetic nanoparticle (MagNP)-mediated gene transfer studies has been increasing for over 10 years because of easy application and success [16]. Keane et al. investigated the role of Fas apoptotic pathway in normal and malignant breast epithelial cell lines and stated that Fas expression in normal cell line is higher than five malignant breast cell lines [17]. When all these cell lines were treated with anti-Fas antibody, it was observed that all of the breast cancer cell lines were resistant to Fas-related apoptosis. Furthermore, viability of MCF-7 cell line was one of the highest and expression of Fas was lower than other cell lines. However, Barnhart et al. reported that MCF-7 cells express high level of Fas but Fas stimulation did not induce apoptosis and NF-κB activation was seen as the major reason of resistance [14]. All these results indicated that breast cancer cells have inactive Fas protein to stimulate apoptosis.

In this study, to provide active Fas expression in breast cancer cells, MagNPs and lipid-based gene transfection were performed. Plasmid DNA (pDNA) that can achieve human Fas expression was transfected to MCF-7 breast cancer cells. Expression of cFLIP and procaspase-8 and the effect of FasL stimulation on apoptosis were investigated. Also transfection success of methods and effects on surface protein were compared.

Materials and methods

pDNA production

Human FAS (green fluorescent protein (GFP)-tagged) plasmid (RG211622) was purchased from OriGene (USA) and reproduced. Briefly, pDNA transfected into Oneshot Match1 *Escherichia coli* competent cell. After selection of positive colonies, the plasmid was propagated in 50 ml LB medium in the presence of ampicillin. Then plasmid was isolated using the GeneJET Plasmid Midiprep Kit (Thermo Scientific, USA) according to the suggested protocol.

Gene transfection

MCF-7 cell line obtained from our university's research center and grown in Dulbecco's modified Eagle's medium (Gibco-ThermoFisher Scientific-USA) supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate and maintained at 37 °C in a CO_2 incubator containing 5% CO_2. The cells were seeded at a density of 10,000 cells per well in a flat bottom 96-well plate and the media refreshed after 24 h. Lipofectamine®2000 (ThermoFisher Scientific-USA) DNA transfection reagent were applied according to the manufacturer's protocol. Briefly, 10 µl DNA–lipid complex (100 ng DNA/well and 0.45 µl Lipofectamine®2000/well) were applied to cells. PolyMag (Chemicell-Germany) DNA transfection reagent were applied for MagNP-mediated gene transfer. A variety amount of MagNPs were applied to compare transfection success between MagNPs and lipid-based transfection. For this purpose, 50 µl DNA–nanoparticle mixtures, which include 100, 200, or 300 ng DNA, were prepared according to the manufacturer's protocol and applied to cells. Immediately cells were placed on MagnetoFactor-96 plate (Chemicell-Germany) for 15 min in an incubator (Supp. Figure-2). After that, cells were incubated for 24 h and GFP expression were imaged by inverted fluorescence microscopy (Model Axio Observer Z1, Zeiss, Germany). To investigate the transfection success, the cells were removed using trypsin and centrifuged for 6 min at 1500 rpm, the medium was discarded, and cells were suspended in 1 ml phosphate-buffered saline (PBS). GFP-expressed cells in PBS were analyzed by flow cytometer (BD Accuri C6-USA).

Apoptosis analysis

According to the results of nanoparticle-mediated gene transfer rate, study continued with 200 ng DNA –nanoparticle mixture and 100 ng DNA–lipid mixture. After adding DNA–reagent mixture, cells were incubated for 24 h and imaged by inverted fluorescence microscopy. Then 20 ng soluble FasL (sFasL)-human (Enzo-Switzerland) were added to each well and apoptosis analyses were performed at 6, 24, and 48 h after sFasL addition. The cells were removed and centrifuged for 6 min at 1500 rpm, medium was discarded, and the cells were suspended in 100 µl medium. Then 100 µl of Muse® Annexin V and dead cell reagent (Merck-Germany) were added to the suspended cells and incubated for 20 min in the dark at room temperature. Apoptosis and dead cell rate were analyzed by Muse® Cell Analyzer (Merck-Germany). Study groups were designed as control (nothing applied), control+sFasL (soluble FasL added), gene transfection by Lipofectamin (Lipof+Gene), Lipof+Gene+sFasL, gene transfection by MagNP (MagNP+Gene), and MagNP+Gene+sFasL.

Western blot (WB) analysis

Study groups each consisting of 20 wells were prepared as explained above and harvested 48 h later. Cell pellets were suspended in 100 µl RIPA lysis buffer and vortexed 4 times. After freezing–thawing at −80 °C, they were centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant (whole-cell protein extract) protein concentration were quantitated by Qubit Fluorometer (ThermoFisher-USA). Then equal amounts of protein (15 µg/ml) were mixed with 2× Laemmli buffer and loaded to gel. After running in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% non-fat dry milk at +4 °C overnight. Membranes were immunoblotted with the primary antibodies FLIP (D5J1E) and cleaved caspase-8 (Asp391) (both obtained from Cell Signaling, The Netherlands) at +4 °C for 1–4 h. Subsequently, the membranes were washed five times with Tris-buffered saline-Tween 20 and then incubated with secondary antibody immunoglobulin G conjugated with horseradish peroxidase (1:2000 Cell Signaling, The Netherlands) at room temperature for 1 h. After that, ECL Western blotting detection reagents (ThermoFisher-USA) were used for imaging. The band density was quantified by using the ImageJ software (National Institutes of Health, USA). The protein level was normalized to β-actin and Lamin B1.

Statistical analysis

Sidak multiple comparison test was performed with the GraphPad Prism software for statistical analysis.

Results

GFP expression levels were analyzed by flow cytometer and transfection rate were obtained as percentage (Fig. 1). MagNP-based transfection with 100 ng DNA (NP-100) were significantly smaller than any other transfection groups ($p < 0.01$). There is no significant differences between Lipofectamin transfection and MagNP-based transfection with 200 ng (NP-200) and 300 ng (NP-300) pDNA, whereas transfection success with NP-200 is slightly higher than both Lipofectamin and NP-300 transfection. Therefore, experiments were continued by using NP-200 and Lipofectamin.

GFP expression of transfected cells were observed in 96-well plate by inverted fluorescence microscopy (Fig. 2).

Apoptosis studies were performed at 6, 24, and 48 h after FasL addition. Apoptosis profile of all groups are shown in Supp. Figure-3. Percentage of live cells, total apoptotic cells (late apoptotic+early apoptotic), and only late apoptotic

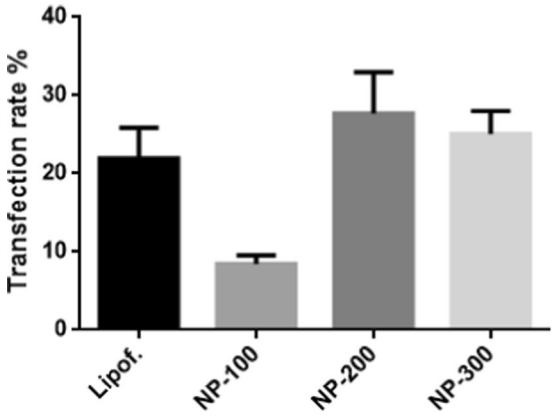

Fig. 1 Comparisons of transfection rates by using Lipofectamin (Lipof.) and magnetic nanoparticles. Magnetic nanoparticles with 100, 200, and 300 ng pDNA (NP-100, NP-200, and NP-300) ($n = 3$, mean ± standard deviation (SD))

rates were analyzed in Fig. 3. Early apoptosis rates was not shown because they are very small compared to late apoptosis. The percentage of live cells are significantly higher than the total apoptotic cells for control, control +Fas, and MagNP (without pDNA) at any time interval ($p < 0.0001$). However, the percentage of total apoptotic cells are significantly higher than the live cells for the MagNP +Gene, MagNP+Gene+FasL, Lipof+Gene, and Lipof +Gene+FasL groups at 24 h. The statistical significance remains the same for the MagNP+Gene and MagNP+Gene +FasL groups ($p < 0.001$) and slightly decreases for the Lipof+Gene group ($p < 0.05$) at 48 h, whereas there is no significant differences for the Lipof+Gene+FasL group.

Effect of transfection reagents (without pDNA) on total apoptosis were also investigated for 24 h. Total apoptosis of MagNP was 13.06 ± 5.78, MagNP+FasL was 13.46 ± 2.26, Lipof was 19.56 ± 1.41, and Lipof+FasL was 26.65 ± 2.22 for 24 h. Early apoptosis of Lipof+FasL was noticeable with the rate of 11.66 ± 1.20. (% mean ± SD, $n = 3$)

WB results

Although the same numbers of cells were studied and proteins were quantitated by Qubit Fluorometer to decide the appropriate amount of samples for loading to gel, amount of both B-actin and Lamin-B1 of the Lipofectamin groups were smaller than the control and nanoparticle groups (Fig. 4). However, amount of both B-actin and Lamin-B1 were similar for the control and nanoparticle groups. There is no caspase-8 production in the control and control+FasL groups, whereas full-length caspase-8, which is approximately 60 kDa, was clearly seen in the gene-transfected groups (Fig. 4).

In literature, weight of c-$FLIP_L$ is described as 55–60 kDa, which is seen as c-$FLIP_L$ in Fig. 4. However, unknown

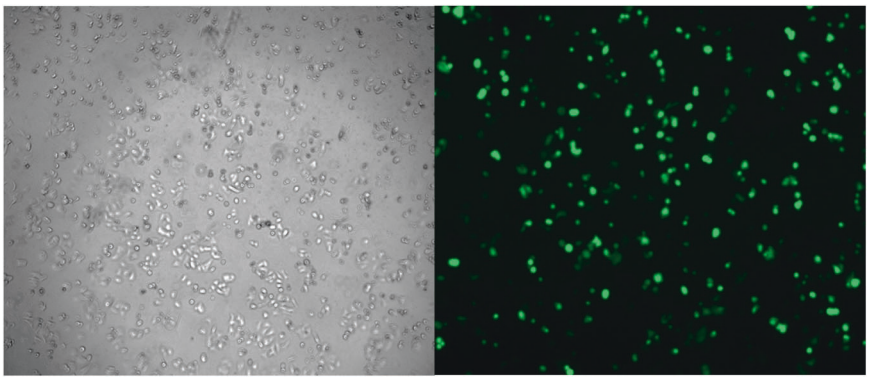

Fig. 2 Light and fluorescence microscopic images of human FAS (GFP-Tagged) plasmid-transfected cells. ×5 magnification

protein at 48 kDa appeared and it was named as c-FLIP$_L$-X here. Antibody manufacturer's WB gallery indicates the unknown protein between 40 and 50 kDa, which was obtained from immunoprecipitation of FLIP from KARPAS-299 cell extracts. c-FLIP$_S$ were observed at 25 kDa for the control and MagNP groups, whereas no c-FLIP$_S$ were seen for the Lipofectamin groups.

After quantifying the WB images by the imageJ software, two different calculations were performed. First, data of each samples (Actin, caspase-8, c-FLIP, and Lamin-B1) were compared with the normalized value of control. In other words, values of sample were divided by the normalized value of control group (Fig. 5a1, a2). Then values of actin were taken as reference and the values of each group (control, Lipof, MagNP, etc.) for caspase-8 and c-FLIP were divided by values of actin. Same calculations were performed by taking Lamin-B1 as reference and the graph of the results are shown in Fig. 5b1, b2.

Discussion

Cell surface death receptor CD95 (Fas) and its activation by ligand CD95L (FasL) are largely illuminated by the studies performed in T-cells as an extrinsic apoptotic pathway [4, 18]. This pathway does not work in cancer cells because high c-FLIP concentration compete with caspase-8 to join DISC structure in the intracellular side of CD95. However, variety of protein variations can be seen on different cell types. For instance, expression of Fas in MCF-7 cells was reported as lower compared to other breast cancer cell lines, such as ZR75-1, MB-231, MB-415, and MB-468 ve SKBr3 [17]. Gene therapy can be a solution for both cancer therapy and understanding the resistance on Fas apoptotic pathway. MagNP-mediated gene transfer is a useful technique as a non-viral transfection method. In this study, NP-200 had higher transfection rate than NP-300, which indicated that the increase in the amount of NP and DNA does not affect transfection rate linearly. The transfection rate of NP200 and the total apoptosis of MagNP+Gene and +FasL groups at 6 h after FasL addition was close to each other which indicates that the transfected pDNA induced the apoptosis. However, total apoptosis rates, which is higher than gene transfection rates, increased with the rise of incubation time at both 24 and 48 h after FasL addition. Effect of transfection reagent without DNA can explain the increase on total apoptosis at 24 h, and slightly higher apoptosis rate of the Lipof+Gene+FasL group compared to the others supports this idea, because Lipof+FasL (without DNA) addition resulted in 26% apoptosis at 24 h. Nevertheless, transfection reagent Lipof may have lost its effect on apoptosis at 48 h because the apoptosis rate of the Lipof groups are lower than the results at 24 h. On the other hand, apoptosis rate of the MagNP groups at 48 h were slightly higher than the results at 24 h, which made us think that cells might continue to uptake NP-DNA structure by endocytosis.

FasL addition has no significant changes on cell viability and apoptosis rate for control group. The rate of late apoptosis is much higher than that of early apoptosis for any time interval. Total apoptosis of gene-transfected cells are significantly higher than the control groups ($p < 0.001$). Theoretically, FasL addition should start apoptosis pathway; however, total apoptosis of only gene-transfected cells was almost the same as the gene-transfected+FasL-added cells. Apoptosis of MagNP-mediated gene-transfected cells is 65% and that is significantly higher than Lipofectamin-mediated gene transfection at 48 h. Increased number of untransfected cells in the Lipofectamin groups might cause relatively low apoptosis because dead cell rates were similar for the both Lipofectamin and MagNP groups or Lipofectamin might suppress transfected cell proliferation.

It is clear that all breast cancer cell lines express Fas protein, whereas they are resistant to Fas-mediated apoptosis and NF-κB activation was seen as major reason of the resistance [14, 17]. Activation of NF-κB related with high expression of c-FLIP and increased c-FLIP concentration may reduce procaspase-8 level in DISC formation, which cause resistance of Fas-mediated apoptosis [1, 3, 5, 10, 14]. WB analysis indicated that there is no caspase-8 production

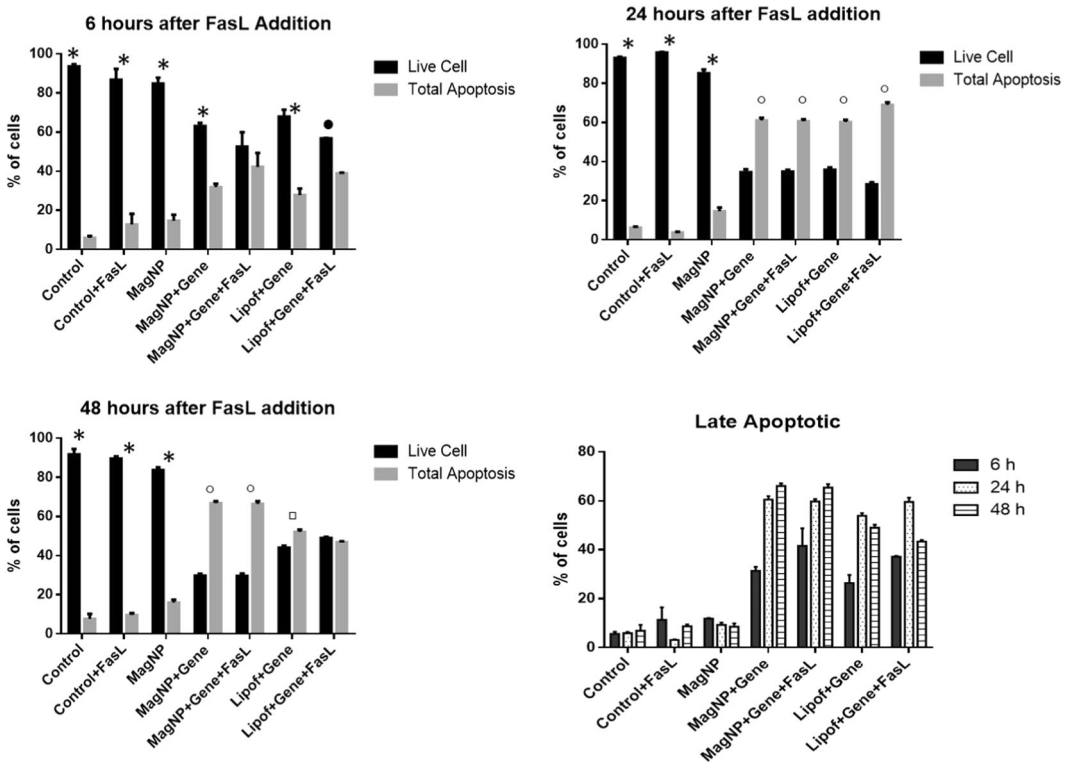

Fig. 3 Live and total apoptosis (late+early apoptosis) at 6, 24, and 48 h after FasL addition. Late apoptotic cells were analyzed at 6, 24, and 48 h. Asterisk (*) represents the percentage of Live cells that are significantly higher than the percentage of total apoptotic cells for each group ($p < 0.0001$). Closed circle (•) represents the percentage of Live cells that are significantly higher than the percentage of total apoptotic cells for each group ($p < 0.05$). Open circle (∘) represents the percentage of total apoptotic cells that are significantly higher than the percentage of Live cells for each group ($p < 0.0001$). Open square (▫) represents the percentage of total apoptotic cells that are significantly higher than the percentage of Live cells for each group ($p < 0,05$). ($n = 3$, mean ± standard error of mean (SEM))

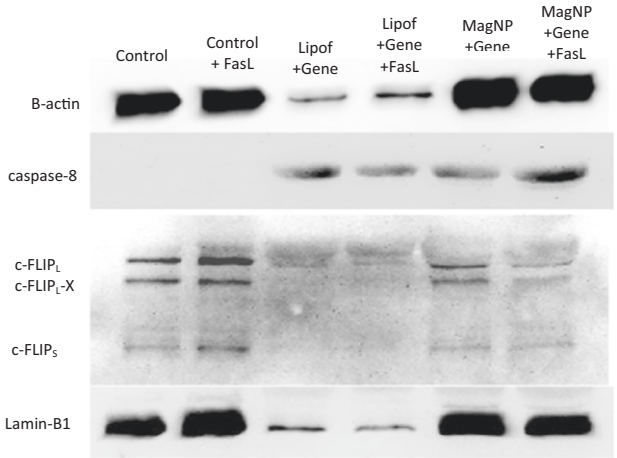

Fig. 4 Western blot images of all groups

in MCF-7 breast cancer cell lines, and similarly, Dupont et al. showed no caspase-8 in the WB images of untreated MCF-7 cells [19]. On the other hand, the gene-transfected cells definitely produce procaspase-8 protein and the results of apoptosis studies indicated that procaspase-8 expressed by pDNA can trigger apoptosis pathway. Although FasL binding was supposed to start apoptosis pathway, there is no significant differences on apoptosis rate on FasL addition. It was reported that Fas and FasL can be produced by the same cell in some types of cancer [10–12], which may cause same apoptosis rates for the FasL added and non-added groups in this study. However, FasL addition increases caspase-8 production significantly in MagNP-mediated gene-transfected cells compared to other gene-transfected group that is related to transfection success.

All experiment groups produced c-FLIP$_L$ and c-FLIP$_S$. Although WB images indicated that there was very small amount of c-FLIP production for the Lipofectamin used groups, calculations made by comparing actin and lamin-B1 show that c-FLIP$_L$ rates for the Lipofectamin used groups were significantly higher than other groups (Fig. 5b2). This was caused by the fact that normalized actin and lamin-B1 values were smaller than one for the Lipofectamin groups and it was placed in the denominator in the calculation that resulted in high rates of c-FLIP$_L$. However, calculated values of both c-FLIP$_L$ and c-FLIP$_S$ production for MagNP used groups were smaller than for the control groups. Both normalized and calculated values indicated that FasL addition increased the amount of c-FLIP$_S$. Moreover, caspase-8 level was approximately the same for both the

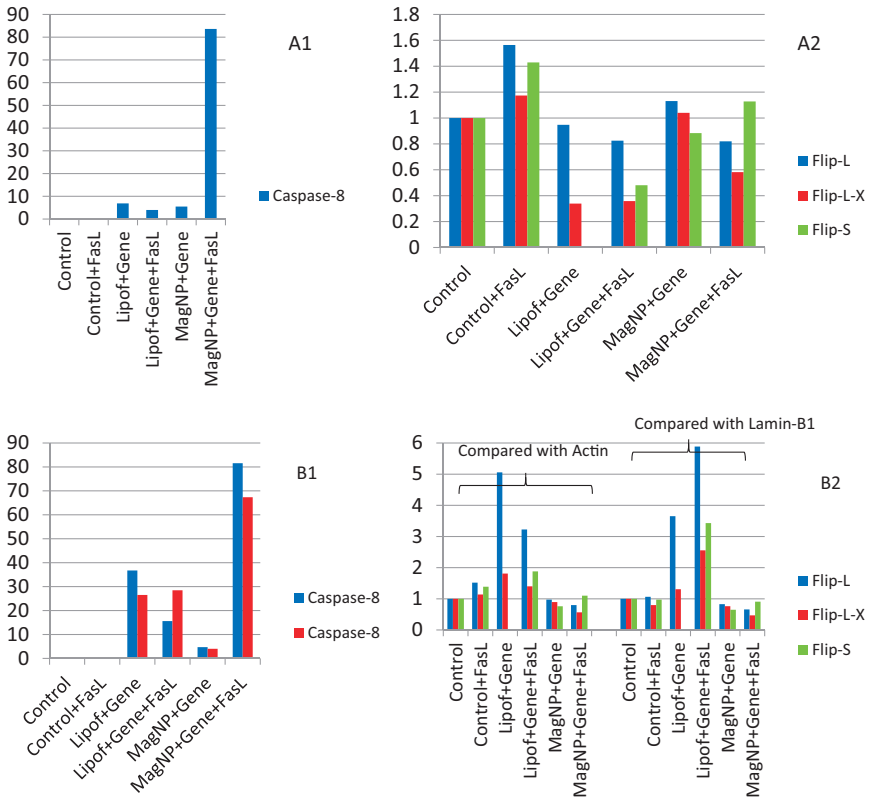

Fig. 5 Quantified values of western blot results. **a1**, **a2** Graphs of normalized values of caspase-8 and c-FLIP compared with control (control group normalized to 1). **b1**, **b2** Graphs of calculated values of caspase-8 and c-FLIP compared with B-actin and lamin B1

Lipof+Gene and Lipof+Gene+FasL groups when lamin B1 was used as a loading control. However, when actin was used as a loading control caspase-8 level in the Lipof+Gene group was significantly higher than the Lipof+Gene+FasL group (Fig. 5b2). All these results indicates that Lipofectamin affects the expression of actin and lamin B1. On the other hand, MagNP has no such effects on cells, therefore it is more reliable for transfection studies. An unknown protein at 48 kDa also was detected and it was named as c-FLIP$_L$-X because its molecular weight is close to c-FLIP$_L$. It is thought that c-FLIP$_L$-X could be p43FLIP, which is the initial caspase-8 product of c-FLIP$_L$ and play a role in activation of NF-κB [18]. Similar protein expression can be seen on WB images of KARPAS-299 cell extracts at antibody manufacturer's web page, which has not been named yet.

In conclusion, MagNP-mediated gene transfer is a successful non-viral method for transfection and it does not affect the expression of other cell proteins, such as beta actin and lamin-B1. According to the result of this study, MCF-7 breast cancer cell line does not express caspase-8 but c-FLIP$_L$, which indicates that the raised c-FLIP$_L$ concentration in cytosol inhibits apoptosis. Therefore, monoclonal antibody therapy to activate CD95 (Fas)-associated caspase-8 apoptosis pathway in MCF-7 cells is not possible. However, pDNA, which is able to express human Fas, can express procaspase-8 when transfected to MCF-7 cells and activate caspase-8 apoptosis pathway. Monoclonal antibody FasL interaction indicated a slight increase in apoptosis rate, whereas a significant rise in caspase-8 expression in transfected cells. Human Fas pDNA and the method applied in this study can be a weapon against cancer.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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