Treatment of human tumor cells by combine gene therapy harnessing plasmids expressing human tumor necrosis factor alpha and bacterial cytosine deaminase suicide gene*

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We have assessed the effect of combine cancer gene therapy with exogenous human tumor necrosis factor alpha (*hTNFα*) and *Escherichia coli* cytosine deaminase (*CD*) suicide gene on two human breast adenocarcinoma cell lines MDA-MB-361 and SK-BR-3. Transfection of a plasmid containing *hTNFα* under the control of a hybrid promoter resulted in expression of *hTNFα* gene *in vitro*. Transduction of retroviral plasmid containing bacterial cytosine deaminase led to the expression of cytosine deaminase in adenocarcinoma cell lines as well. The significant increase in apoptotic cells and decrease of cell proliferation in both tumor cell lines was observed using combination treatment with *hTNFα* expression plus *CD*/5-FC suicide system. Corresponding data were generated by MTT cell proliferation assay and by flow cytometric analysis. The presence of both genes after transduction of retroviral vector containing *CD* and transfection of *hTNFα* plasmid was confirmed by PCR and RT-PCR. The additive cell killing effect due to the presence of bacterial *CD* and *hTNFα* gene after activation of non-toxic prodrug was observed. Whether the bicistronic vector containing both therapeutic genes improve the therapy need to be assessed in the future.

Key words: cancer gene therapy, retroviral vector, bacterial cytosine deaminase, tumor necrosis factor alpha, apoptosis

Cytosine deaminase gene (*CD*) containing vector with subsequent application of 5-fluorocytosine (5-FC) is considered as a potent suicide cancer gene therapy approach. Cytosine deaminase is absent in mammalian cells, but present as a 52-kDa protein in bacteria, fungi, and yeast. It catalyses the hydrolytic deamination of cytosine to uracil and analogically 5-fluorocytosine to 5-fluorouracil, a potent cytostatic compound. The final outcome of 5-fluorouracil intracellular metabolism is the inhibition of DNA and RNA synthesis, resulting in cell death [1]. 5-fluorocytosine is relatively non-toxic drug acting as a prodrug in cells expressing cytosine deaminase. Its therapeutic efficacy after conversion to 5-fluorouracil (5-FU) is promoted not only by its cytostatic action but by the bystander effect as well. This is due to its release from cytosine deaminase-expressing cells across tumor cell membranes and diffusion into neighboring non-transduced cells. In the addition, the anti-neoplastic effect of 5-FU has been shown to enhance radiation response of tumor cells and is often used as a sensitizing agent in clinical praxis [2–6]. *CD*/5-FC gene therapy combined with the use of ionizing radiation therefore represents a new approach for cancer treatment by selectively sensitizing of tumor cells. Further killing enhancement was described combining two enzyme/prodrug systems: cytosine deaminase/5-fluorocytosine and thymidine kinase/ganciclovir [7–9]. This double suicide gene therapy approach enhanced cytotoxicity and increase radio-sensitivity.

In our experiments we intend to improve cancer gene therapy by combination cytosine deaminase/5-fluorocytosine suicide system with hTNFα expression. Tumor necrosis factor alpha (TNF-α) is a key cytokine secreted by T-cells and activated macrophages. TNF-α is involved in inflammation, immunity and cellular organization. The mechanism by which hTNFα leads to cell death is complex. Systemic administration of hTNFα due its cytotoxicity is rather limited but local tumor killing action is quite effective [10]. Molecu-

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lar mechanism of hTNFα starts with its homotrimerization, binding to two distinct cell surface receptors: tumor necrosis factor receptors TNFR1 and TNFR2 that are independently expressed on different cell types [11, 12]. TNFR1 contains a cytoplasmic death domain, which is important in initiating apoptosis and other signaling pathways after hTNFα-binding. The binding of hTNF-α to its receptor results in receptor trimerization and aggregation of the death domain. This allows another steps [13, 14], which activates caspase cascade ending with apoptosis [15–18].

In the present study, we have investigated the effectiveness of the combine cancer gene therapy using suicide system bacterial cytosine deaminase/5-fluorocytosine, with expression of *hTNFα*. The enhanced killing effect on cancer cell lines was proved by the measurement of MTT proliferation rates and by detection of induced apoptosis by means of flow cytometry.

Material and methods

Expression vectors. pORF-hTNFα (InvivoGen, San Diego, USA) contains an intron-less open reading frame of the human *TNFα* downstream of the EF-1α/HTLV hybrid promoter. The control plasmid pORF-minus-hTNFα was constructed as described previously by digesting pORF-hTNFα with SgrA I and Nhe I to remove the hTNFα fragment [10]. Schematic maps of the plasmids are presented in Figure 1. Green fluorescent protein expression plasmid pCIneoGFP was used for determination of the transfection efficiency (kindly provided by Geoff Margison and Joanna Libby, Paterson Institute for Cancer Research, Christie Hospital, Manchester).

Retroviral vectors. pJH17 bicistronic retroviral vector containing bacterial cytosine deaminase and gene for neomycin resistance separated by IRES sequence, both genes expression are under the LTR promoter/enhancer region [19].

Schematic maps of the plasmids are presented in Figure 1.

Tumor cell lines. The following cell lines were used: MDA-MB-361 and SK-BR-3 (both human breast adenocarcinoma). The cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

Cell transfection. 10^5 cells were transiently transfected with 1 µg of either pORF-hTNFα or pORF-minus-hTNFα using GeneJuice transfection reagent (Novagen) according to the manufacturer's recommendation. Plasmid pCINeoGFP expressing green fluorescent protein was used to optimize the ratio between amount of DNA and transfection reagent. The transfection medium was replaced with fresh medium after 14 hours.

Retroviral infection of cancer cell lines. One day before infection, $2x10^5$ recipient cells were plated in 60 mm Petri dishes. For transduction of MDA-MB-361 and SK-BR-3 cells the medium filtered through a 0.45 µm filter from retrovirus producing cells GP+envAm12/pJH17 [19] was used. Infection was done by replacing the medium of the recipient cells with 2 ml of medium with viral particles supplemented with Polybrene 8 µg/ml. Selection medium contained G418 was applied twenty four hours after virus infection. The resistant cell lines MDA-MB-361/CD and SK-BR-3/CD were obtained after 14 days of G418 selection.

Analysis of hTNFα gene and CD gene presence by PCR. Total cellular DNA from transfected cells was obtained by phenol/chloroform extraction. The specific primers for PCR detection of *hTNFα* and *CD* genes were used to confirm the presence of the transgene.

Nucleotide sequence of primers:

TNFα forward:

5`CCGGAATTCACCATGAGCACTGAAAGCATG 3'

TNFα reverse:

5`ATAGTTTAGCGGCCGCGAATTCCTCACAGGGCAATG 3'

hTNFα 702bp fragment

CD forward:

5`AAAGGATCCGTGTCGAATAACGCTTTACAA 3'

CD reverse:

5`TTTGTCGACTCAACGTTTGTAATCGATGGC 3'

CD 1302bp fragment.

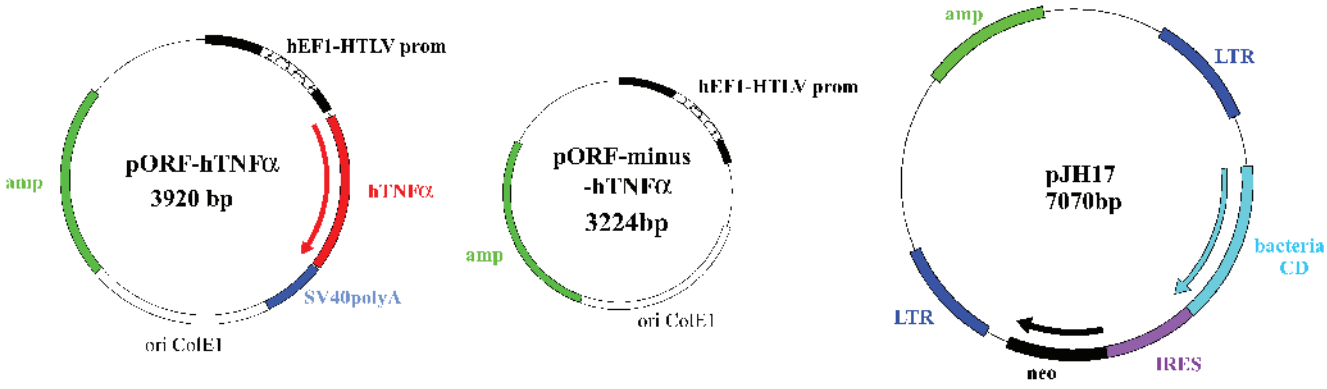


Figure 1. Schematic maps of expression plasmid vectors pORFhTNFα, control plasmid pORF-minus-hTNFα and retroviral vector pJH17.

Analysis of hTNF alpha and bacterial cytosine deaminase expression by RT-PCR. Total cellular RNA was isolated using RNeasy Mini Qiagen kit reagent preparation protocol, followed by the DNAase treatment. The lack of DNA contamination in cleaned RNA samples was confirmed by PCR. RT reaction was carried out at 42 °C for 60 minutes, with 1 μg of RNA and random hexamer primers. Obtained cDNA was used for PCR reactions with *TNFα*, bacterial *CD* specific primers. *GAPDH* primers were used as an internal control.

Sequence of primers:
TNFα forward: 5`CAGAGGGAAGAGTTCCCCAG 3'
TNFα reverse: 5`CCTTGGTCTGGTAGGAGACG 3'
TNFα 326bp fragment
CD forward:
5`AAAGGATCCGTGTCGAATAACGCTTTACAA 3'
CD reverse: 5`CAGGCTGTTTTCAGTTATGG 3'
CD 151bp fragment
GAPDH forward: 5`GAAGGTGAAGGTCGGAGTC 3'
GAPDH reverse: 5`GAAGATGGTGATGGGATTTC 3'
GAPDH 226bp fragment

Biological effects of hTNFα and CD/5-FC therapy in transfected cells. Apoptotic and necrotic cells were quantified after transfection with plasmids and after three or five-day cultivation with or without addition of 5-fluorocytosine, by flow cytometry (EPICS ALTRA) using the Annexin V-FITC kit (Immunotech) according to the manufacturer's recommendation. In addition the MTT assay was conducted to determine the cell survival rate. Fourteen hours after transfection, 10^4 cells per well were plated into 96-well plates, 6 hours later the triplicates of cells were treated with different concentrations of 5-FC (Roche) and continued to be cultured for five days, when the medium was replaced by MTT mixture. The absorbance of the cells was measured after 3 hours at a wavelength of 490 nm using an ELISA plate reader. The survival rate was expressed as A/B x 100% minus background, where A is the absorbance value from the experimental cells and B is absorbance of the control cells (MDA-MB-361/CD and SK-BR-3/CD transfected with control plasmid pORF-minus-hTNF without prodrug treatment).

Statistical analysis. Quantitative variables are described as mean ± standard error of the mean, both expressed as percentages. Comparison between percentages was performed with the Student's t-test. A p value <0.05 was taken as the criterion of statistical significance.

Results

In order to study combine effect of bacterial cytosine deaminase (*CD*) suicide gene with action of exogenous human tumor necrosis factor alpha (*hTNFα*), the MDA-MB-361 and SK-BR-3 (human breast adenocarcinoma) cells containing integrated bacterial cytosine deaminase suicide gene were transfected with expression *TNFα* gene plasmid. The presence of those two genes was confirmed by PCR assay (Fig. 2).

The expression of cytosine deaminase was found similar by RT-PCR assay in all tested *CD* transduced cells transfected with control plasmid pORF-minus-hTNFα and also in all cells transfected with pORF-hTNFα (Fig. 3). There was no difference in *TNFα* gene expression in *TNFα* transfected tumor cell lines used in the experiments, despite difference in their transfection efficiency.

Transfection effectiveness was monitored by flow cytometric estimation of percentage of GFP positive cells after transfection with GFP expressing plasmid. The transfection efficiency was estimated to be 18.7±2% for the MDA-MB-361/CD cells, 38.2±0.7% for the SK-BR-3/CD cells (Fig. 6, 7).

The MTT assay was used to determine the cell survival rate. The survival rate of cytosine deaminase gene containing cells was significantly lower in a dose 5-FC-dependent manner ($p<0.05$) than in corresponding parental cells (MDA-MB-361, SK-BR-3). The cells expressing both bacterial cytosine deaminase and *TNFα* gene (MDA-MB-361/CD+hTNFα, SK-BR-3/CD+hTNFα) were shown the most sensitive to 5-FC with a half lethal dose significantly lower. The *TNFα* gene expression in MDA-MB-361/CD+hTNFα cells lead to 60% cell survival with half lethal dose 6.1 μg/ml of 5-FC after its addition, whereas for the MDA-MB-361/CD cells the half lethal dose was 50 μg/ml of 5-FC, being

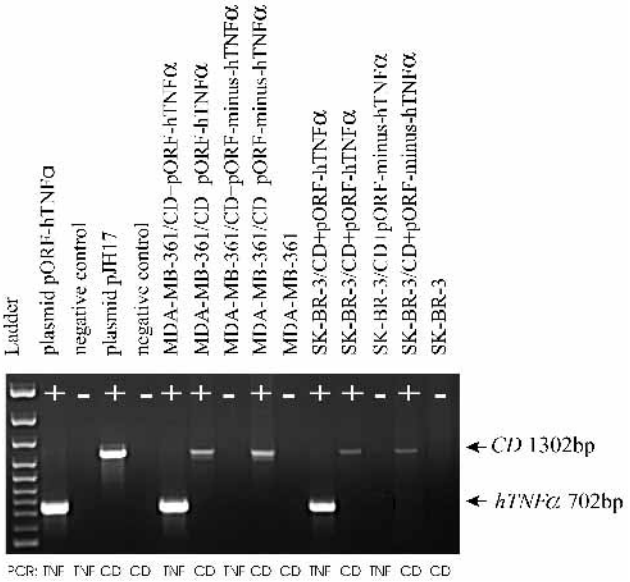


Figure 2. Detection of exogenous *hTNFα* gene and bacterial cytosine deaminase gene in transfected and transduced cells by PCR. Lane 1 – DNA ladder; lane 2 – PCR reaction with plasmid pORF-hTNFα, lane3 – PCR reaction mixture without DNA, lane 4 – PCR reaction with plasmid pJH17, lane 5 – PCR reaction mixture without DNA, lane 6, 7 – MDA-MB-361/CD cells transfected with pORF-hTNFα lanes 8, 9 – MDA-MB-361/CD transfected with control plasmid pORF-minus-hTNFα lane 10 – MDA-MB-361 lanes 11, 12 – SK-BR-3/CD cells transfected with pORF-hTNFα lanes 13, 14 – SK-BR-3/CD cells transfected with control plasmid lane 15 – SK-BR-3.

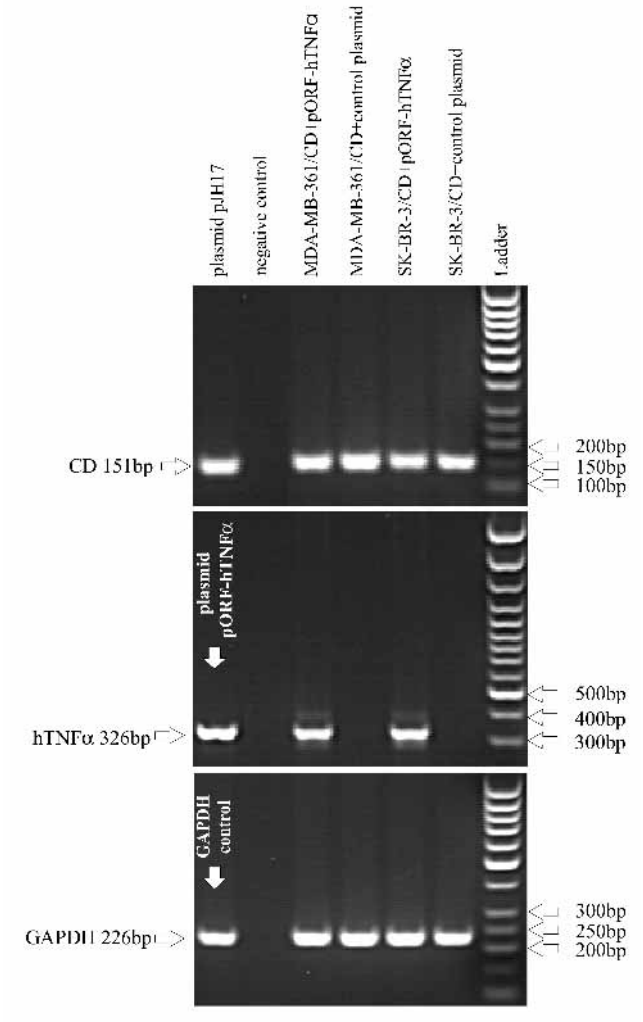


Figure 3. Detection of exogenous *hTNFα* and cytosine deaminase expression in transfected and transduced cells by RT-PCR. Lanes are in following order: RT-PCR control reaction with pJH17 or pORF-hTNFα plasmid or GAPDH cDNA; RT-PCR reaction mixture without cDNA; MDA-MB-361/CD cells transfected with pORF-hTNFα; MDA-MB-361/CD cells transfected with control plasmid pORF-minus-hTNF; SK-BR-3/CD cells transfected with pORF-hTNFα; SK-BR-3/CD cells transfected with control plasmid.

8.2-times higher (Fig. 4). 5-fluorocytosine half lethal dose for SK-BR-3/CD+hTNFα cells was found 10 μg/ml, for SK-BR-3/CD cells more than 1000 μg/ml, being more than 100 times higher (Fig. 5). The survival rate of parental MDA-MB-361 and SK-BR-3 cells was not affected by the presence of 5-FC.

To quantify the apoptotic portion of the treated cells by flow cytometry, annexin V/PI dual staining was performed measuring the cells 72 or 144 hours after transfection in all variations of treatments (after single either *CD*/5-FC or *TNFα* gene treatment; and after combine *CD*/5-FC plus *TNFα* treatment). Results obtained by flow cytometry have shown that in the MDA-MB-361/CD cells treated with 100 μg/μl of 5-FC ratio of apoptotic to alive cells slightly increased. There was an additional increase ($p<0.05$) in number of apoptotic cells when MDA-MB-361/CD cells were transfected with *TNFα* containing plasmid and treated with 5-FC (Fig. 6).

Breast adenocarcinoma SK-BR-3/CD cells were sensitive to single 5-FC-treatment (Fig. 7). The percentage of apoptotic cells in SK-BR-3/CD cell line without 5-FC 144 hours after transfection was 16.2% and 30.5% in SK-BR-3/CD *TNFα*-expressing cells without 5-FC treatment. Single 5-FC-treatment of SK-BR-3/CD cells with 150 μg/ml or 500 μg/ml lead to apoptosis in range of 35.9–46.7 percent. In combine treatment of cells (SK-BR-3/CD plus *TNFα*) in the same 5-FC concentration, the amount of apoptotic cells increased to 37.7% and 58.4%, respectively (Fig. 7). There was difference in proportion of alive and apoptotic cells in SK-BR-3/CD cell line if we compare period of time 72 hours and 144 hours after pORF-hTNFα transfection for concentration 0 and 500 μg/ml of 5-FC (data not shown).

Both the MTT and flow cytometry results of combined suicide gene therapy (*CD*/5-FC with expression of *hTNFα*) demonstrated the enhanced killing effects to two different human breast carcinoma cells.

Discussion

Therapeutic gene transfer affords a clinically feasible and safe approach for cancer therapy but a more effective technique is needed to improve clinical outcomes. Combined transfer of therapeutic genes with different modes of action may be means to this end.

Insertion of genes that activate prodrugs to produce cytotoxic compounds in tumor cells is considered as a potential therapeutic strategy for cancer therapy. Several suicide genes are used for this purpose. The advantage of cytosine deaminase as a product of suicide gene is that intra-cellularly produced toxic drug kills not only the target tumor cells, but by bystander effect also neighboring cells. Experiments were done to see whether another additional tumor killing effect can by obtained by insertion of cytotoxic cytokine into the tumor cells. The systemic application of human tumor necrosis factor alpha is not possible for extended period of time in patients, due to numerous side effects. However, expression of human tumor necrosis factor alpha in tumor tissue might help to kill tumor cells locally. Previously we have shown that the human tumor necrosis factor alpha expression plasmid applied in the tumor tissue caused hemorrhagic necrotic lesions [10].

This study demonstrated that prodrug activation by bacterial cytosine deaminase and simultaneous expression of *TNFα* in two human breast cancer cell lines MDA-MB-361 and SK-BR-3 yields an anticancer effect that is enhanced, comparing with the effect observed with single-prodrug treatment or cytokine expression. Cancer gene therapy using

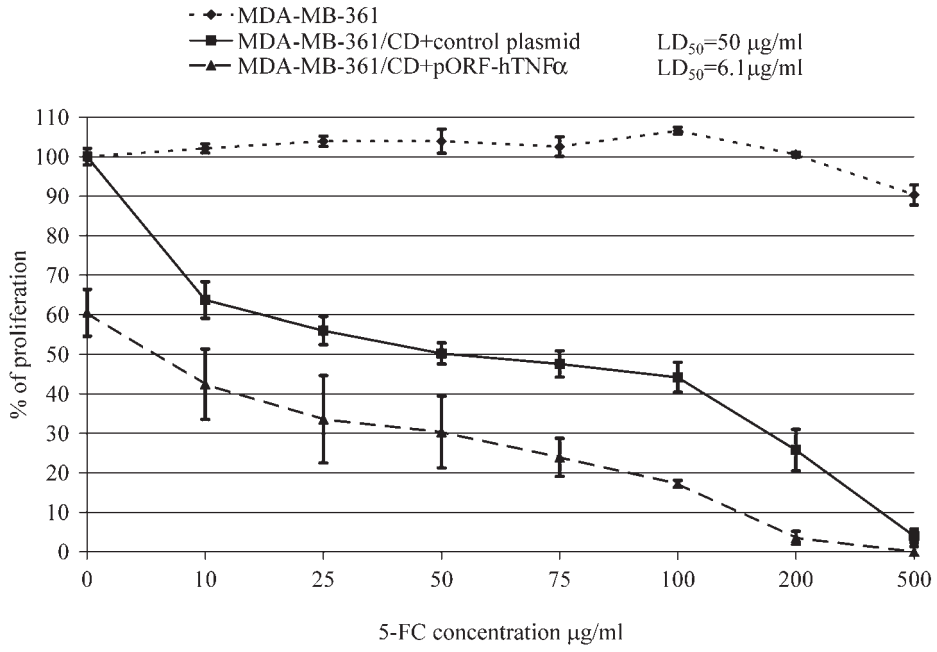


Figure 4. Dose-dependent 5-day survival of MDA-MB-361 cells, MDA-MB-361/CD cells transfected with control plasmid pORF-minus-hTNFα, and MDA-MB-361/CD cells transfected with pORF-hTNFα cultured in different concentration of 5-fluorocytosine. Cell proliferation was detected by MTT assay and expressed as percentage.

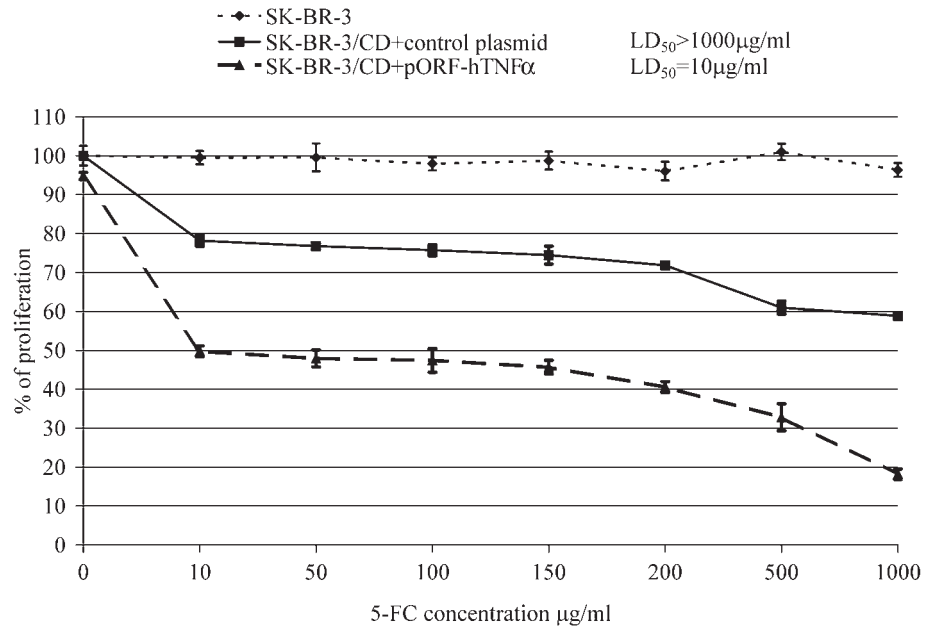
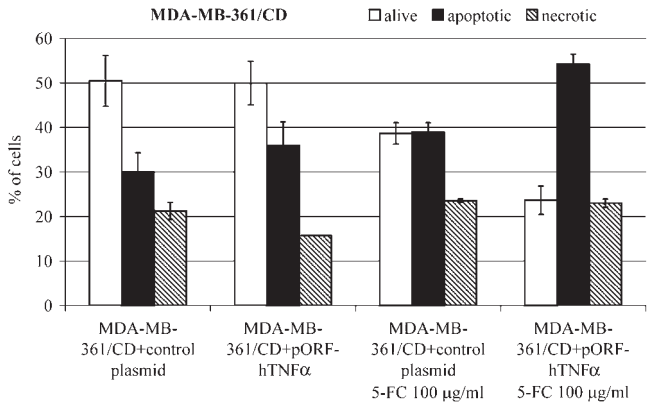


Figure 5. Dose-dependent 5-day survival of SK-BR-3 cells, SK-BR-3/CD cells transfected with control plasmid pORF-minus-hTNFα, and SK-BR-3/CD cells transfected with pORF-hTNFα cultured in different concentration of 5-fluorocytosine. Cell proliferation was detected by MTT assay and expressed as percentage.

retroviral vector containing *CD* gene combined with 5-FC treatment offers two attractive features for cancer therapy. One is the preference of retrovirus vectors to transduce actively dividing cells. The second advantage is that non-transduced tumor cells can be eliminated by means of bystander effect involving free diffusion of 5-FU from cytosine deaminase expressing cells.

There have been no reports up to date dealing with the CD/5-FC suicide gene therapy in combination with hTNFα cytokine expression neither *in vitro* nor *in vivo* system. Similar results were reported [20] showing that TRA-8, the monoclonal antibody that binds to death receptor 5 – one of two death receptor of TRAIL, with combination of adenoviral vector directed CD enzyme/5-FC prodrug therapy produced an additive cytotoxic effect in cancer cells *in vitro* and *in vivo*.

Our recent results [21] and even studies of others [22, 23] reported that the combination of *TNFα* and *Herpes simplex* thymidine kinase/ganciclovir suicide gene system produced a more potent therapeutic benefit in different tumor models *in vitro* and *in vivo*. The efficiency of combine or single *TNFα*/*HSVtk* gene therapy was found dependent on type of tumor cells used for the treatment approach [10, 24–28]. In our experiments double-gene therapeutic strategy using cytosine deaminase/5-fluorocytosine suicide gene therapy with combination of *TNFα* cytokine expression was found more efficient, leading to greater proportion of tumor cells dying by apoptosis. These data indicate that combined treatment with suicide gene therapy and *TNFα* cytokine expression provides a promising approach for cancer therapy.



Figue 6. Apoptosis caused by 5-FC treatment of the MDA-MB-361/CD cells transfected with control plasmid pORF-minus-hTNFα, and the MDA-MB-361/CD cells transfected with pORF-hTNFα. Apoptotic cells were detected by flow cytometry using double annexin-V-FITC and PI staining after 5-days of 5-FC treatment. *Transfection efficiency was 18.71±2% as was found by GFP expression.

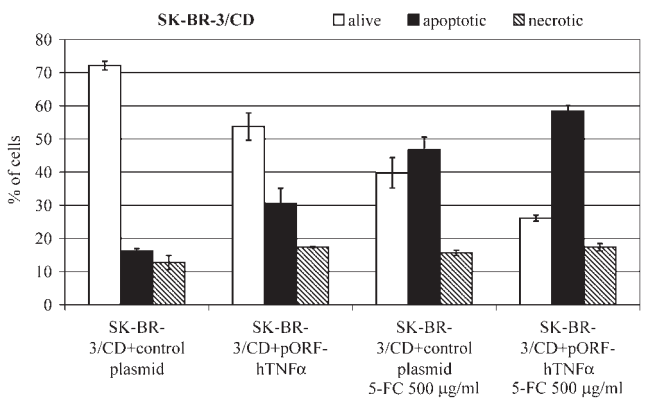


Fig. 7. Apoptosis caused by 5-FC treatment of the SK-BR-3/CD cells transfected with control plasmid pORF-minus-hTNFα, and the SK-BR-3/CD cells transfected with pORF-hTNFα. Apoptotic cells were detected by flow cytometry using double annexin-V-FITC and PI staining after 5-days of 5-FC treatment. *Transfection efficiency was 38.2±0.72% as was found by GFP expression.

Based on the results obtained *in vitro*, we intent to construct novel retroviral vector for local administration containing both suicide cytosine deaminase gene and human tumor necrosis factor gene.

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