Tumor targeted gene therapy with plasmid expressing human tumor necrosis factor alpha *in vitro* and *in vivo*[*]

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Received April 4, 2005

We have assessed the effect of exogenous human tumor necrosis factor alpha (hTNFα) in three human cancer cell lines; MDA-MB-361 (breast adenocarcinoma), HCT 116 (colon carcinoma) and 8-MG-BA (glioma). *In vitro* transfection of a plasmid containing hTNFα under the control of a hybrid promoter resulted in expression of hTNFα gene in all three cell lines and secretion into the culture medium was seen with MDA-MB-361 cells. Flow cytometric analysis showed a significant increase in apoptotic and necrotic cells in MDA-MB-361 and to a lesser extent in HCT 116 cells. Increased apoptosis was confirmed by an increase in pro-caspase 3 activation. No effects of hTNFα expression were seen in 8-MG-BA cells. Intratumoral delivery of the hTNFα expression plasmid into MDA-MB-361 tumor xenografts grown in nude mice caused hemorrhagic tumor necrosis. This strategy may be a simple and promising gene therapy approach to the treatment of some human tumors.

Key words: cancer gene therapy, human tumor necrosis factor alpha, apoptosis, necrosis

Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine that is secreted by macrophages and can elicit a variety of effects: it can cause cytolysis of certain tumor cells; it is a potent pyrogen, causing fever by direct action or by stimulation of interleukin-1 secretion; it is implicated in the induction of cachexia *in vivo*, it can stimulate cell proliferation, induce cell differentiation, and under certain conditions it is responsible for up-regulation of adhesion molecules, thus facilitating the binding of leukocytes to normal vessels [10, 35]. Human TNFα is known to be cytotoxic to variety of human cancer cells *in vitro* [33] and its cytotoxicity has been observed in human tumor bearing mice where hemorrhagic tumor necrosis was induced after intratumoral TNFα delivery [5, 15, 34, 37].

The use of recombinant hTNFα as a systemic cancer therapy is limited due to its toxicity. Systemic hTNFα therapy in combination with chemotherapy has also been disappointing. The discrepancy between the results in the anti-tumor activity of hTNFα observed in human trials and rodent models can be explained by the substantial difference in the tolerability of systemic doses of hTNFα. Thus the maximum tolerated dose of hTNFα in patients has been 5 to 10-fold lower than the effective doses achievable in rodents. This problem of systemic toxicity can be overcome, or reduced, by exploiting hTNFα gene transfer to achieve local anti-tumor therapy [18].

The mechanism by which hTNFα leads to cell death is complex. It is dependent on the presence both of cell-surface receptors and other proteins present in the cells, such as pro-apoptotic and anti-apoptotic factors, on the cell cycle phase, and on other unknown factors. The hTNFα is initially expressed as a 26 kDa membrane bound protein, which is cleaved by the hTNFα converting enzyme that generates the soluble 17 kDa active protein. In the circulation, monomers of hTNFα form homotrimers. The biological activity of hTNFα is realized by binding to two cell surface receptors, Tumor necrosis factor receptors (TNFR) 1 and 2 (55 and 75 kDa molecular weight, respectively) that are independently expressed on different cell types [2, 26]. TNFR1 contains a cytoplasmic death domain, which is important in initiating

[*]The study was supported by the VEGA grants No. 2/3094/24 and 2/3094/25 and by state program "Use of cancer genomics to improve the human population health".

apoptosis and other signaling pathways after hTNFα binding. The death domain-containing receptor is maintained in an inactive state by a 60 kDa protein, named silencer of death domain. Binding of hTNFα to its receptor results in receptor trimerisation and aggregation of the death domain. This allows binding of an intracellular adapter molecule TNFR-Associated Death Domain (TRADD), which interacts with the death domain and subsequently binds various proteins to the activated receptor, thus directing the future fate of the cell. The association of TRADD with Fas-Associated Death Domain (FADD) leads to the induction of apoptosis *via* the recruitment and cleavage of pro-caspase 8 [31, 35]. Caspase 8 activation provides a link between death receptor signaling (extrinsic) and the mitochondrial (intrinsic) pathways of the apoptosis [4, 7, 9, 28]. Apoptosis can be also mediated through the interaction between TNFR1 and an adapter molecule designated as Receptor-Interacting Protein RIP-Associated ICH-1 CED-3 homologous protein with death domain (RAIDD). RAIDD associates with RIP through interaction between death domains, recruits caspase 2 and leads to the induction of apoptosis [6].

In the present study, we have investigated the effectiveness of hTNFα gene therapy both *in vitro* and *in vivo*. Three human cancer cell lines of different origin were transfected with an expression vector containing the hTNFα gene downstream of a hybrid EF-1/HTLV enhancer-promoter. Flow cytometry and other parameters were used to assess the frequency of apoptotic and necrotic cells. Intratumoral delivery of the hTNFα expression plasmid into breast adenocarcinoma tumor xenografts grown in nude mice caused hemorrhagic tumor necrosis.

Material and methods

Expression vectors. pORFhTNFα (InvivoGen, San Diego, U.S.A) contains an intron-less open reading frame of the human TNFα downstream of the EF-1/HTLV hybrid promoter. The control plasmid (pORF-minus-hTNFα) was constructed by digesting pORF-hTNFα with SgrA I and Nhe I to remove the 3200 bp hTNFα fragment and religating the resulting vector using an adapter (5'-CCGGCGGATCCG-3' annealed to 5'-CTAGCGGATCCG-3'). Fidelity was verified by BamH I digestion (site introduced in linker) and by PCR amplification with hTNFα primers designed to detect intron-less gene (sense primer:
5'CCGGAATTCACCATGAGCACTGAAAGCATG3'
and antisense primer:
5'ATAGTTTAGCGGCCGCGAATTCCTCACAGGGCAATG3').
Schematic maps of the plasmids are presented in Figure 1. Green fluorescent protein expression plasmid pCIneoGFP was used for determination of the transfection efficiency.

Cell lines. The cell lines used were: MDA-MB-361 breast adenocarcinoma cells, HCT 116 human colon carcinoma cells, and 8-MG-BA human glioma cells. 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.

Transfection of cells. 10^5 cells were transiently transfected with 1 µg of either pORF-hTNFα or pORF-minus-hTNFα using GeneJuice transfection reagent (Novagen) or Effectene (Qiagen) according to the manufacturer's recommendation. The optimal ratio between amount of DNA and transfection reagent was obtained using plasmid pCINeoGFP (green fluorescent protein downstream of the cytomegalovirus immediate-early enhancer/promoter, kindly provided by Geoff Margison and Joanna Libby, Paterson Institute for Cancer Research, Christie Hospital, Manchester). The transfection medium was replaced with fresh medium after 4 hours.

Analysis of hTNFα alpha gene presence by PCR. Total cellular DNA from transfected cells was obtained either by phenol/chloroform extraction or by Trizol isolation. The primers described above for PCR detection of hTNFα were designed in such a way to exclude detection of endogenous hTNFα and to confirm the presence of the transgene.

Detection of caspase 3 activation in cells and hTNFα protein secretion into culture fluid by Western blot analysis. Protein extracts were prepared in a cell lysis solution (1% SDS, 10 mmol TrisHCl, pH 7.5, 1 mmol PMSF, 1% Sigma protease inhibitor cocktail). The sonicated extract was centrifuged at 20000 x g for 30 minutes at 4 °C and protein concentration was determined by the Lowry protein assay. Cell extracts were separated by 12% SDS-PAGE electrophoresis, blotted to nitrocellulose membranes and the presence of caspase 3 shown using an anti-caspase 3 monoclonal antibody (Ab-2, Calbiochem). Culture medium (2 ml) from 10^5 transfected cells was concentrated using an Amicon ULTRA-4 Centrifu-

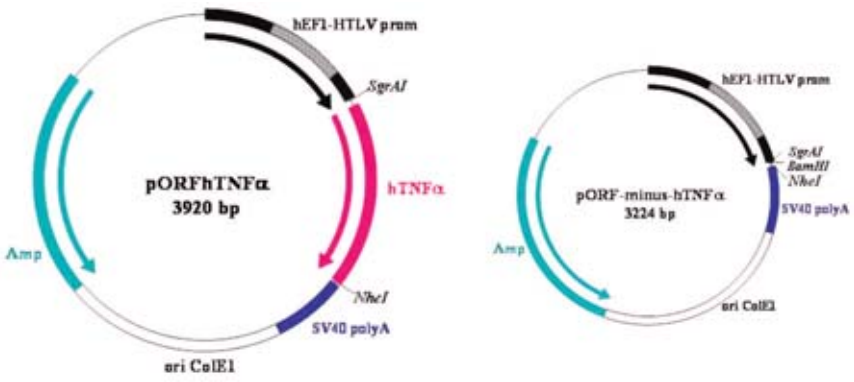

Figure 1. Schematic maps of pORFhTNFα and pORF-minus-hTNFα.

gal Filter Device 5000 MWCO and the concentrate was subjected to SDS polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane and probed using an anti-hTNFα monoclonal antibody TNFα (Ab-1, Calbiochem).

Biological effects of hTNFα in transfected cells. Apoptotic and necrotic cells were quantified by flow cytometry (FACS) using the Annexin V-FITC kit (Immunotech).

Athymic nude mice and tumor xenografts. Nude mice (BALB/c-nu/nu) used in the experiments were bred and housed under pathogen-free conditions and fed *ad libitum*. A total of 10^7 MDA-MB-361 cells in 0.1 ml serum-free tissue culture medium were injected under aseptic conditions subcutaneously into 6-week old female mice in two dorsal sites. When the tumor volume reached ≥50 mm^3 the tumors were inoculated intra-tumorally with pORF-minus-hTNFα (mock treatment) or pORFhTNFα either as naked DNA (15 μg per tumor) or 5 μg of DNA together with 7 equivalents of linear 25-kDa polymer of polyethylenimine (Polysciences). Tumor volumes were determined using calipers and the ratio of tumor volumes for treated (T) and mock treated (MT) tumors grown on the same animal (T/MT) was taken as a measurement of the efficiency of the treatment. A ratio of less than 50% was considered a significant therapeutic effect.

Results

The presence of the hTNFα transgene in the transfected MDA-MB-361, HCT 116 and 8-MG-BA cells was shown by PCR amplification using primers that were able to detect the intron-less hTNFα gene (Fig. 2).

Western blot analysis confirmed that hTNFα protein was secreted into the culture medium of the MDA-MB-361 cells at 16 and 18 hours after transfection increasing somewhat at 24 hours after transfection (Fig. 3). In control experiments, the cells that were transfected with the expression plasmid from which hTNFα was removed (pORF-minus-hTNFα) proliferated without any signs of toxicity. In addition, no growth inhibition was seen in cells that were transfected with pORF-minus-hTNFα. In contrast, pORFhTNFα-transfected MDA-MB-361 and HCT 116 cells started to die shortly after transfection. The proportion of cells alive, apoptotic, and necrotic at several time intervals after transfection detected by Annexin V-FITC kit were determined by flow cytometry (Fig. 4) and the results are presented in Table 1 (MDA-MB-361), Table 2 (HCT 116) and Table 3 (8-MG-BA).

MDA-MB-361 cells started to die by apoptosis 2 days after transfection, and this was followed by increasing numbers of necrotic cells on the third day. Six days after transfection few live cells were present. The HCT 116 cells started to die one day after transfection, and necrotic cells gradually increased the next day. The HCT 116 cells have doubling times of ~20 hours and those not affected by hTNFα quickly outgrew the dead cells. On the other hand, the human glioma cell line 8-MG-BA was found to be resistant to hTNFα, despite a higher transfection efficiency.

The changes induced in the course of cell growth following transfection were easily recognized by cell morphology and cell density. Representative results are shown in Figure 5.

After transfection MDA-MB-361 cells started to die by apoptosis. The proportion of apoptotic cells increased during the first 6 days in the culture, followed by increase of necrotic cells at 3rd day after transfection. The cells do not grow rapidly in culture; therefore the increase of apoptotic cells may be due to the release of hTNFα into the culture medium. Long term cultivation of transfected MDA-MB-361 classified by flow cytometry as alive cells lead to continuation of cell death as documented in Figure 5/F. The rest of the cells do not proliferate, despite the fact that some cells still remain attached even after nine weeks of culture.

In order to establish if cell death was due to the induction of apoptosis via caspase activation, pro-caspase 3 and its activated form was determined in the cell lysates by western blot analysis involving the appropriate loading controls. There was an increase in pro-caspase 3 activation implicating hTNFα expression as a cause of apoptosis (Fig. 6).

To examine the effects of gene therapy *in vivo*, we gave single intra-tumoral injections of pORFhTNFα into one of two dorsal bilateral MDA-MB-361 xenografts and the control plasmid (pORF-minus-hTNFα) into the other. The biological effects of treatment were evaluated by measurement of tumor volume, by macroscopically visible morphological changes, and by histo-pathological findings. Tumor volume was measured and a positive response, as defined by T/MT<50%, was seen in 65% of the TNFα-treated tumors (26 tumor bearing nude mice were treated). The most striking effect of the tumor-targeted hTNFα gene delivery was the induction of hemorrhagic tumor necrosis (observed as black colored tumors). There were no such effects on the other (control plasmid injected) tumor on the same mouse. The same effects were seen using naked DNA or DNA complexed with polyethylenimine. The macroscopically visible morphological effects on the treated tumor correlated with pronounced changes in the histology of the tumors. Tumor necrosis was mostly multifocal affecting large areas of the tumor and together with hemorrhage involved about two third of the tumor volume (Fig. 7). There was no change in body weight of the animals, indicating no systemic hTNFα-mediated toxicity. Repeated inoculation of plasmid into tumor correlated with the efficacy of the therapy. The administration of the hTNFα plasmid DNA did not have any effect on proliferative activity as measured by the Ki67 labeling index. We observed neither significant anti-apoptotic (Bcl-2), nor apoptotic activity (Bax) in either treated or control tumors, indicating that the intrinsic apoptotic pathway was not affected by the therapy (Fig. 7).

Discussion

There have been numerous attempts to use cytokines such as hTNFα, IL-1 and IL-6 for cancer treatment [17, 13]. How-

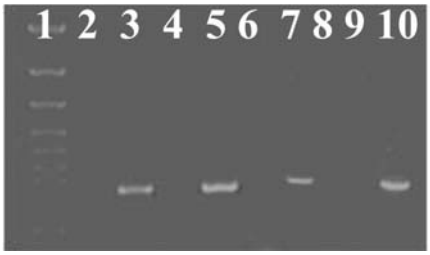

Figure 2. PCR detection of exogenous hTNFα gene in transfected cells. 1 – DNA ladder; 2 – MDA-MB-361 transfected with pORF-minus-hTNFα; 3 – MDA-MB-361 transfected with pORFhTNFα; 4 – HCT 116 cells transfected with pORF-minus-hTNFα; 5 – HCT 116 cells transfected with pORFhTNFα; 6 – 8-MG-BA cells transfected with pORF-minus-hTNFα; 7 – 8-MG-BA cells transfected with pORFhTNFα; 8 – empty track; 9 – PCR mixture without DNA; 10 – PCR with pORFhTNFα plasmid.

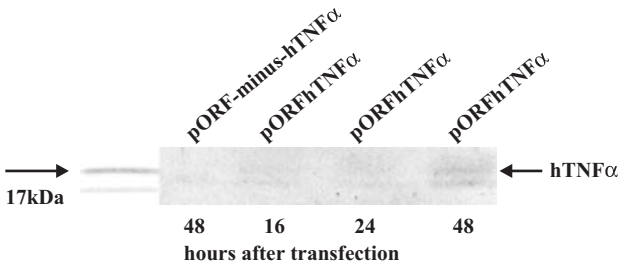

Figure 3. Secretion of hTNFα into the culture fluid. Serum-free culture medium from pORFhTNFα and pORF-minus-hTNFα transfected MDA-MB-361 cells was concentrated and Western blotted using an anti-hTNFα monoclonal antibody.

Table 1. The proportion of alive, apoptotic, and necrotic MDA-MB-361 cells after transfection

Hours after transfection	pORF-minus-hTNFα*			pORFhTNFα*		
	Alive %	Apoptotic %	Necrotic %	Alive %	Apoptotic %	Necrotic %
24	81.93 ± 7.02	8.72 ± 2.75	9.1 ± 4.3	83.21 ± 0.59	8.27 ± 1.29	8.14 ± 0.89
48	81.63 ± 4.87	12.44 ± 3.55	5.72 ± 1.44	62.47 ±1.36	29.58 ± 0.46	7.53 ± 0.96
72	77.71 ± 6.7	15.68 ± 5.31	6.36 ±1.33	46.04 ± 6.94	26.22 ± 3.7	27.39 ± 3.1
144	86.4 ± 3.14	8.71 ± 3.52	4.8 ± 0.82	21.33 ± 5.87	66.55 ± 5.32	12.19 ± 3.31

*the efficiency of transfection measured by GFP expression was 8.05±1.4%.

Table 2. The proportion of alive, apoptotic, and necrotic HTC 116 cells after transfection

Hours after transfection	pORF-minus-hTNFα*			pORFhTNFα*		
	Alive %	Apoptotic %	Necrotic %	Alive %	Apoptotic %	Necrotic %
24	84.4 ±1.62	7.8 ± 0.31	7.43 ± 1.3	69.95 ± 2.5	24.21 ± 1.65	5.7 ± 0.91
48	80.66 ± 3.00	9.14 ±0.96	10.12 ± 2.06	50.93 ± 3.76	27.29 ± 1.45	21.71 ± 2.6
72	80.97 ± 0.69	8.28 ± 0.66	10.95 ±1.24	71.89 ± 2.12	14.56 ± 0.95	14.07 ± 0.99

*the efficiency of transfection measured by GFP expression was 8.0±1.49%.

Table 3. The proportion of alive, apoptotic, and necrotic 8-MG-BA cells after transfection

Hours after transfection Transfection reagent	pORF-minus-hTNFα*			pORFhTNFα*		
	Alive %	Apoptotic %	Necrotic %	Alive %	Apoptotic %	Necrotic %
48 Effectene	61.97 ± 6.88	10.42 ± 3.2	27.45 ± 3.68	65.89 ± 4.58	9.51 ± 3.57	24.48 ± 0.912
48 GeneJuice	52.72±2.50	8.65 ± 3.79	39.11 ± 1.84	58.48±1.64	15.10 ± 1.74	26.90 ± 0.74
144 GeneJuice	51.32±2.61	29.63 ± 3.57	19.16 ± 1.04	47.34 ± 4.80	34.09 ± 4.64	18.70 ± 0.30

*the efficiency of transfection measured by GFP expression was 17.7±0.43% for GeneJuice and 37.8±2.13% for Effectene.

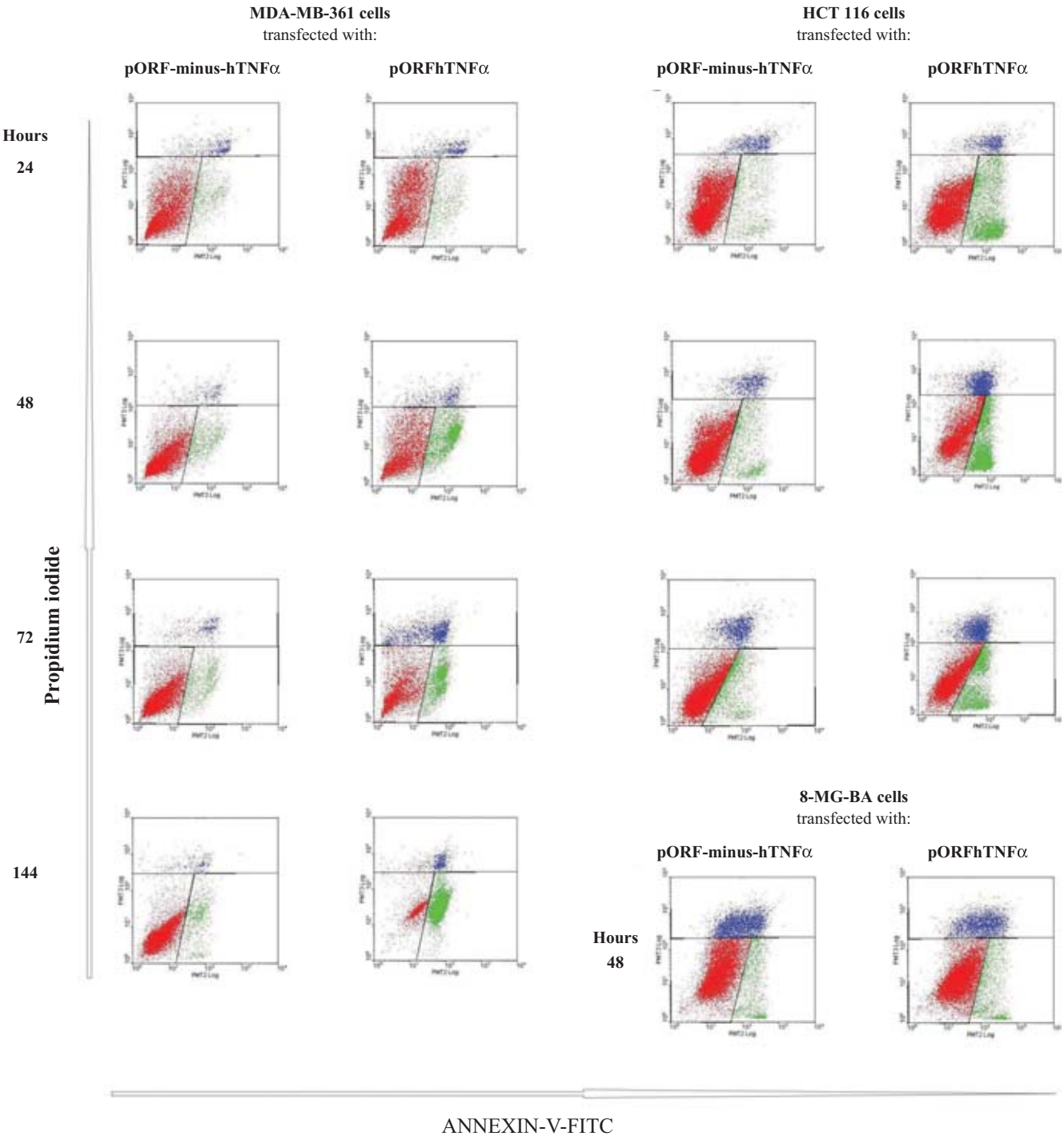

Figure 4. Flow cytometry analysis of transfected cells in the course of hTNFα action using double Annexin-V-FITC and propidium iodide staining. MDA-MB-361, HCT 116 and 8-MG-BA cells were transfected with pORFhTNFα or the control plasmid pORF-minus-hTNFα. After the times indicated, the cells were trypsinized, washed and treated with annexin-V-FITC and propidium iodide. Red spots are living cells, green spots are cells in apoptosis, and blue spots are cells undergoing necrosis.

ever, therapy using systemic administration of hTNFα protein, for example, is hampered by its high normal tissue toxicity [1, 30]. Local administration of hTNFα could be a strategy to reduce systemic toxicity tissue only [14, 16, 22, 29]. The ability of hTNFα to induce hemorrhagic tumor necrosis after local application depends on the molecular char-

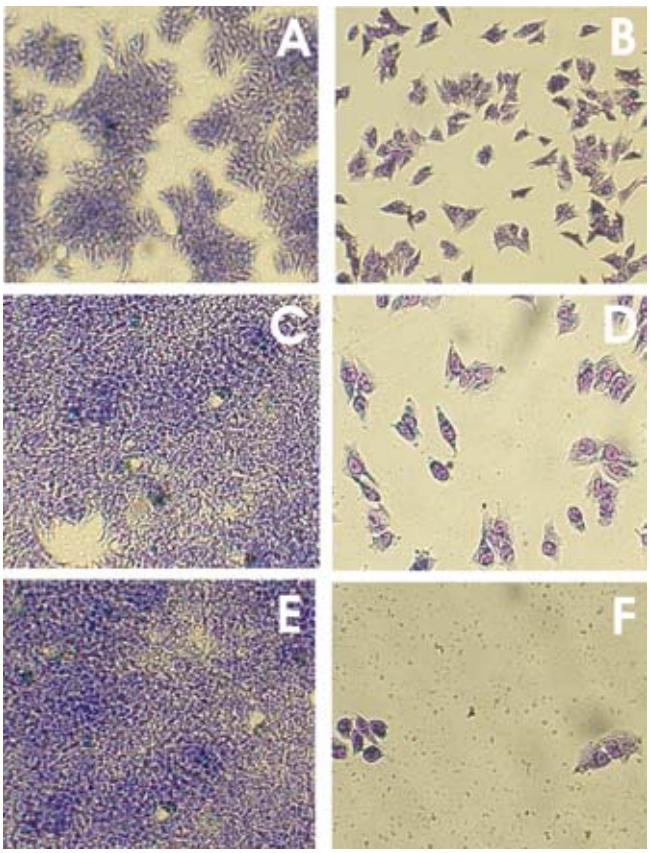

Figure 5. Induction of cell death in transfected cells. MDA-MB-361 were transfected with pORFhTNFα or pORF-minus-hTNFα and stained with Giemsa-Romanowski dye 96, 144, and 240 hours later. Photographs were taken at 100x magnification. A, C, E – MDA-MB-361 cells transfected with pORF-minus-hTNFα. B, D, F – MDA-MB-361 cells transfected with pORFhTNFα. A, B – 96 hours after transfection; C, D – 144 hours after transfection; E, F – 240 hours after transfection.

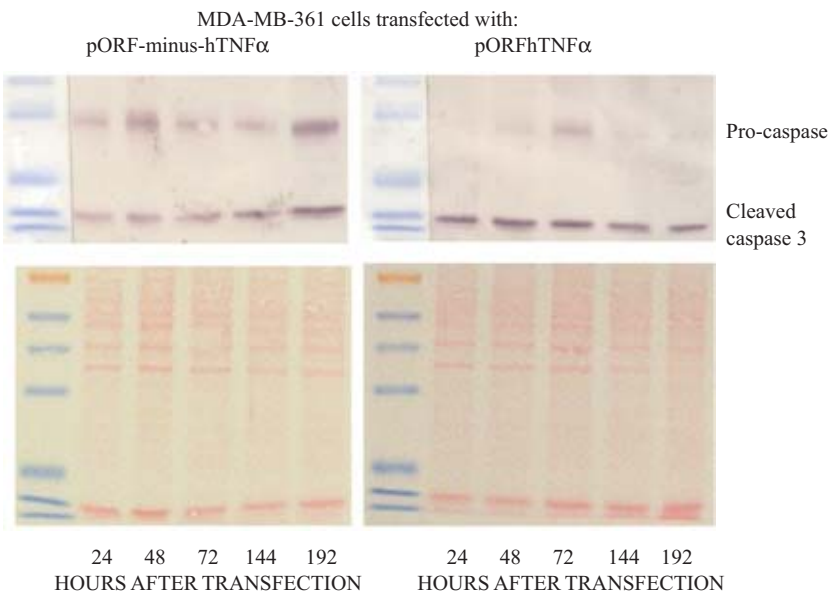

Figure 6. Activation of pro-caspase 3 in the course of hTNFα action in MDA-MB-361 cells. Lysates of pORF-minus-hTNFα or pORFhTNFα transfected cells were analyzed by immunoblotting with monoclonal antibody recognizing both pro-caspase 3 and cleaved caspase 3.

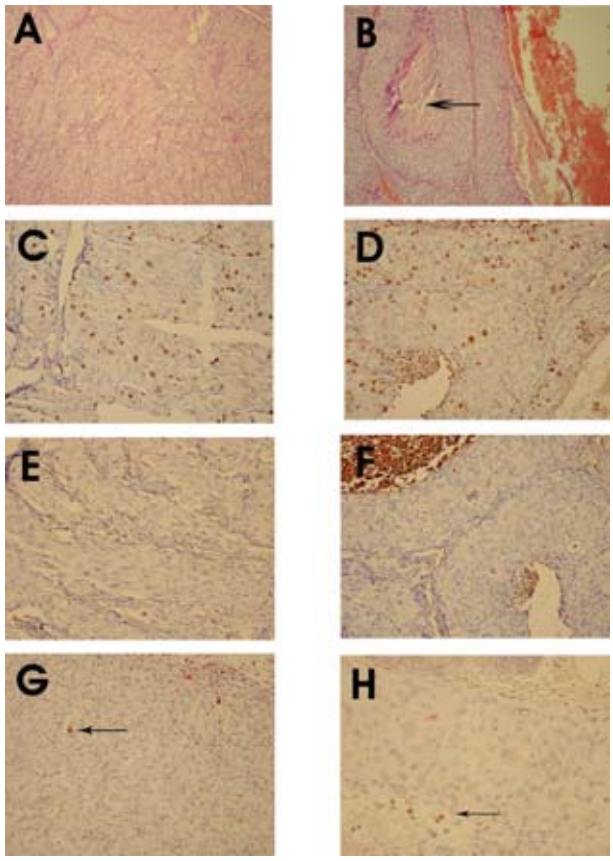

Figure 7. Induction of necrotic lesions by inoculation of plasmid pORFhTNFα in MDA-MB-361 tumors grown in nude mice; histological findings. On each nude mouse tumors were grown in two locations on the back. One tumor was treated with pORF-minus-hTNFα (A, C, E, G) and the other with pORFhTNFα (B, D, F, H). Sections from both tumors were stained with hematoxylin-eosin (A, B), or immunoassayed for detection of Ki67 (C, D), Bcl-2 (E, F), or Bax protein (G, H), respectively and evaluated with light microscopy. A – tumor composed of solid sheets of pleomorphic cells with high mitotic activity. No necrosis was observed. B – necrosis (arrow) and hemorrhage encompassing up to two thirds of the tumor (A and B – magnification 200x). C and D – proliferation index (Ki-67) shows high activity (approximately 40%) with no visible difference between control and pORFhTNFα groups. E and F – no Bcl 2 protein was detected (C – F – magnification 400x). G and H – apoptotic marker Bax showed only scarce positivity (less than 1%, arrow) (G – magnification 200x, H – magnification 400x).

acteristics of the tumor cells. Thus the biological effect of hTNFα is dependent on the presence of receptors on the cell membrane and on other factors present in the cells including pro-apoptotic and anti-apoptotic proteins, on the cell cycle phase, and on other unknown factors. Besides the general toxicity of hTNFα, it also has particular affinity for proliferating tumor endothelial cells, where it can produce vascular damage by inducing endothelial cell apoptosis [23, 25, 32]. In contrast, quiescent vessels in normal tissues are not influenced [19, 24]. However, the biological effect of hTNFα critically depends on the dose: low doses are angiogenic, whereas high doses are antiangiogenic [8].

An alternative approach is to use inoculation of a hTNFα expression vector directly into tumor to overcome the systemic toxicity of hTNFα. In the present study, we have found that human breast adenocarcinoma cells (MDA-MB-361) and colon carcinoma cells (HCT 116) are sensitive to hTNFα. TNFα-mediated apoptosis was responsible for this effect in MDA-MB-361 cells as supported by caspase 3 activation. In agreement with previous observations in the absence of apoptotic stimuli, these cells expressed high basal levels of pro-caspase 3 together with its activated form [39]. Following hTNFα treatment, pro-caspase 3 was cleaved to the active form, i.e. with time, the amount of pro-caspase 3 decreased and active caspase 3 level was elevated.

It has been reported that human tumor cells expressing high levels of apoptotic signaling also contain high levels of inhibitors of apoptotic proteins (IAP). This upregulated IAP expression counteracts the high basal caspase 3 activity in tumor cells. Since we detected apoptosis in MDA-MB-361 and HCT 116 cells by expressing exogenous hTNFα, this suggests that apoptotic pathways in these cells are not blocked.

Human glioma cells (8-MG-BA) were found to be resistant to the effects of expression of exogenous hTNFα despite a high transfection efficiency. This might be due to a dysfunction in apoptotic pathways. It was reported that the glioma cell lines U-105 MG, U-251 MG and SF-767 are resistant to apoptosis and that this was caused by a block in the apoptotic signaling pathways between caspase 8 and caspase 3 [12]. It is also known that glioma cells, as well as normal astrocytes, are generally resistant to Fas-induced apoptosis [27, 38]. Because of the similarity between Fas-induced caspase cascade activation and the hTNFα signaling pathway leading to apoptosis, it is likely that a similar molecular mechanism is involved in the resistance of 8-MG-BA. It might alternatively, or additionally be that the observed resistance of 8-MG-BA cells to hTNFα action is caused by the intracellular retention of the cell surface receptor, TNFR 1. Resistance to hTNFα therapy was also reported to be caused by NF-kappaB activation [3, 21, 36]. The processes involved in the observed resistance of 8-MG-BA cells to hTNFα action remain to be elucidated. However, 8-MG-BA cells were found to be sensitive to gene therapy using a cDNA vector expressing the wild-type p53 protein [11] and restoration of wild-type p53 functions in mutant cells increases their susceptibility to the cytotoxic action of tumor necrosis factor [20]. Therefore a combination gene therapy with hTNFα and wild-type p53 may be a potential strategy to sensitize hTNFα-resistant tumors to the cytotoxic action of this cytokine.

In summary, local intratumoral application of a vector expressing hTNFα seems promising way for treatment tumors that are sensitive to this type gene therapy, and this approach should be considered for clinical trial, possibly in combination with stimulators or potentiators to improve its efficiency.

We wish to thank Mrs. M. DUBROVCAKOVA for technical assistance and Miss V. FRIVALSKA for maintenance of animals.

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