Transduction of Cytokine Genes Into Human Colon Carcinoma Cells

Ulrike Stein, Wolfgang Walther, Robert H. Shoemaker*

Background: Multidrug resistance can be a major obstacle to successful cancer chemotherapy and is often associated with increased expression of the mdr1 (also known as P-glycoprotein) gene. Some of the proteins produced by the body's immune system, i.e., cytokines such as tumor necrosis factor-α (TNF) and interleukin 2 (IL-2), have been shown to modulate multidrug resistance. However, cytokines administered by the conventional intravenous method can cause severe side effects. Transduction of cytokine genes into tumor cells constitutes an alternative approach for production and release of the cytokine proteins in the local tumor microenvironment, which may reduce problems of toxicity associated with systemic administration. Purpose: In this study, we investigated the therapeutic potential of a combination of gene therapy and chemotherapy on the basis of cytokine-mediated modulation of multidrug resistance in human colon carcinoma cells. Methods: Human colon carcinoma cell lines HCT15 and HCT116 were transduced with TNF or IL-2 carrying murine leukemia virus (MLV)-based retroviral vectors. Tumor cell clones were analyzed for cytokine expression by reverse transcriptase–polymerase chain reaction (RT–PCR) and by cytokine-specific enzyme-linked immunosorbent assays (TNF-ELISA or IL-2-ELISA). Expression of mdr1 messenger RNA (mRNA) was investigated using RT–PCR, and P-glycoprotein (Pgp) expression was determined by immunoflow cytometry with the monoclonal antibodies MRK16 and C219. The function of Pgp was analyzed by measuring accumulation of the fluorescent drug doxorubicin by flow cytometry. The XTT—(i.e., [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl-2H-tetrazolium hydroxide]—colorimetric cytotoxicity assay was used to determine chemosensitivity of cytokine gene-transfected tumor cells to doxorubicin and vincristine. Statistical significance was determined by the nonparametric Mann–Whitney rank sum test for the flow cytometry experiments (Pgp detection as well as drug uptake assays) and the parametric Student's t test for the chemosensitivity assay (XTT cytotoxicity assay). All P values reported were derived from two-sided statistical tests. Results: Transduction and expression of human TNF and IL-2 in HCT15 and HCT116 human colon carcinoma cell lines were found to reverse multidrug resistance. Both TNF and IL-2 secretion reduced mdr1 expression on the mRNA and Pgp levels (P < 0.0243). This result was associated with enhancement of doxorubicin accumulation within the cells (P < 0.0001). The cytokine-mediated effects on mdr1 expression resulted in increased chemosensitivity of the transduced cells to doxorubicin and vincristine (P < 0.0460). Conclusions and Implications: We show that endogenous expression of cytokine genes in tumor cells and after transduction secretion of the related proteins, such as TNF and IL-2, can modulate multidrug resistance in vitro. This modulation enhances the susceptibility of the cells to the cytotoxic drugs. Our findings suggest the potential value of combined treatment of resistant tumors with gene therapy and chemotherapy. [J Natl Cancer Inst 1996;88:1383-92]

The development of multidrug resistance in human tumors may be a major obstacle to successful cancer chemotherapy (1). The phenomenon of multidrug resistance is often associated with increased expression of the mdr1 (also known as P-glycoprotein) gene, which encodes P-glycoprotein (Pgp) (2). As an energy-dependent efflux pump, this protein is capable of extruding certain drugs from cells, leading to decreased drug concentrations within the cell and reduced efficacy of drugs (3). Since the mdr1 gene appears to represent a major cause of multidrug resistance in tumor cells, mdr1 messenger RNA (mRNA) and Pgp may be important targets in multidrug resistance reversal strategies. Several approaches have been made to influence the activity of Pgp, using substances such as calcium channel blockers, calmodulin antagonists, etc., or to modulate mdr1 gene expression using various cytokines, e.g., tumor necrosis factor-α (TNF), interleukin 2 (IL-2), interferon gamma (IFN γ), or leukoregulin (4-6).

TNF and IL-2 are cytokines that play important roles in immunoregulation and antitumor defense mechanisms. These cytokines have been extensively tested in in vitro and in vivo investigations as well as in clinical trials for immunotherapy for malignant diseases (7-10). However, systemic application of these substances is not without controversy, since both TNF and IL-2 are known to cause severe side effects in patients. Alternative approaches, including gene transfer of cytokine genes into
tumor cells, can be undertaken to circumvent these disadvantages and to potentiate immune response by local cytokine release (11, 12). Both cytokines have been successfully transduced into tumor cells in vitro and in vivo settings, and clinical studies have indicated their antitumor activity and immunostimulatory potential (12, 13). More recently, it has been demonstrated that, among other cytokines, TNF and IL-2 can modulate mdrl expression and enhance cytotoxicity of certain multidrug resistance-related drugs in various tumor cell lines (6, 14). The capability of cytokines to influence the multidrug-resistance phenotype is strongly supported by an earlier study (15) that demonstrated enhanced efficacy of chemotherapeutic drugs in combination with cytokine treatment.

Genes coding for human TNF or IL-2 were introduced into the human colon carcinoma cell lines HCT15 and HCT116. The phenotypes of these cell lines exhibit differing levels of multidrug resistance. The extent of such resistance has been established by analysis of mdrl expression on the mRNA and protein levels as well as on the Pgp functional level, providing a solid basis for the molecular analysis of cytokine-mediated effects in relation to the intrinsic multidrug-resistant phenotype of the tumor cell (16). In the gene transfer experiments, murine leukemia virus (MLV)-based expression vectors were used that constitutively express either TNF or IL-2 under the control of the cytomegalovirus (CMV) promoter. Selected TNF- or IL-2-expressing clones derived from both cell lines were employed to investigate cytokine effects on mdrl gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR), on Pgp expression by immunoflow cytometry using the monoclonal antibodies MRK16 and C219, on Pgp function by measuring doxorubicin uptake in flow cytometry experiments, and on chemosensitivity to the multidrug resistance-related drugs vincristine and doxorubicin. In this study, we investigated the potential of a combination of gene therapy and chemotherapy based on cytokine-mediated modulation of multidrug resistance in human colon carcinoma cells.

Materials and Methods

Overall Approach

We analyzed cytokine gene-transduced colon carcinoma cells to evaluate the potential of endogenously produced and secreted cytokines for reversal of multidrug resistance. Effects of transduced TNF and IL-2 on mdrl gene expression were analyzed on the mRNA and protein levels and their impact on Pgp function was determined in association with in vitro chemosensitivity to multidrug resistance-related drugs.

Construction of the Expression Vector

The expression vector based on the MLV-derived retroviral vector pM3neo was used for insertion of the human TNF complementary DNA (cDNA) or human IL-2 cDNA to create the constructs pM3CMV-hTNF and pM3CMV-hIL2. The plasmid pM3CMV-hTNF was constructed by insertion of the 0.37-kilobase (kb)-BamHI fragment of the CMV promoter from pM13-CMV (provided by M. Strauss, Berlin, Federal Republic of Germany) into the BamHI site of the vector and of the 1.05-kb HindIII fragments of TNF from pM13-hTNF (provided by W. Fiers, Ghent, Belgium) into the HindIII site of the vector so that expression of the TNF gene is driven by the CMV promoter. The plasmid pM3CMV-hIL2 was created by insertion of the 1.0-kb BamHI/HindIII IL-2 fragment from pGEM-IL2 (provided by M. Strauss) into the BamHI/HindIII sites of the vector. The BamHI fragment of the CMV promoter was again taken from the pM13-CMV plasmid for regulation of IL-2 expression and inserted into the BamHI site of the vector.

Cell Lines

The human colon carcinoma cell lines HCT15 (17) and HCT116 (18) were selected from the 61-cells line panel of the National Cancer Institute, Bethesda, MD, which is used extensively for screening of new anticancer drugs. These cell lines have been well characterized, including their expression of resistance-associated genes such as mdrl (16). Both cell lines express the Pgp-mediated type of multidrug resistance intrinsically. However, HCT15 displays a much greater degree of drug resistance compared with HCT116. Cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Steril Systems, Logan, UT) and 5 mm l-glutamine at 37 °C and 5% CO2.

Electroporation

Both the pM3CMV-hTNF and pM3CMV-hIL2 vectors were introduced into the human colon carcinoma cell lines by electroporation (19). Cells were trypano-sinized, suspended in 5 mL medium, centrifuged (900 rpm, 4 °C, 5 minutes), and cell pellets were resuspended in ice-cold phosphate-buffered saline (PBS). The cell concentrations were adjusted to 1 x 10^6 cells/mL, then 20 μg of vector DNA of the appropriate construct was added and cells were kept on ice for 10 minutes, electroporated at 960 μF, 300 V in an ElectroporatorII apparatus (Invitrogen Corp., San Diego, CA), and kept on ice for another 10 minutes. Neomycin-resistant cell clones were selected in 0.8 mg/mL G418 (Life Technologies, Inc. [GIBCO BRL, Gaithersburg, MD]) and expanded for characterization and further experiments.

TNF and IL-2 Enzyme-Linked Immunosorbent Assay (ELISA)

The amounts of secreted TNF or IL-2 in supernatants of transduced HCT15 and HCT116 cell clones were determined using a TNF- or IL-2-specific CytoScreen ELISA kit (BioSource Int. Camarillo, CA). Prior to the assay, fresh medium was added to the cells, and the supernatants were harvested after 24 hours. Aliquots (100 μL) of supernatants from control cells and TNF- or IL-2-expressing clones were used for ELISA to quantify the amounts of secreted cytokines. The assays were performed as recommended by the kit manufacturer. After addition of the chromogen and stop solution, absorbance was measured at 450 nm in a microplate reader. For each assay, standard curves were determined and the TNF or IL-2 values were read from the appropriate standard curve and expressed as cytokine concentration in pg/mL produced by 1 x 10^6 cells in 24 hours.

RNA Isolation and RT-PCR

For isolation of total cellular RNA, cells were washed with 1 mL ice-cold 0.9% sodium chloride solution and then harvested by the addition of 200 μL LiCl/urea solution (3 M LiCl, 6 M urea, Sigma Chemical Co., St. Louis, MO). Total RNA was prepared with the use of the miniprep-RNA protocol (20). RT-PCR was carried out with the use of the Gene Amp RNA PCR Kit (Perkin-Elmer Corp., Foster City, CA, via Roche Molecular Systems Inc., Branchburg, NJ). The RT reaction was performed using 1 μg of miniprep-RNA and random hexamer primers supplied with the kit. For the PCR TNF-, IL-2-, mdrl-, and β-actin-specific primers were used that amplified a 702 base pair (bp)-product for TNF (21), a 462-bp product for IL-2 (21), a 167-bp product for mdrl (22), and a 316-bp product for β-actin (16).

The RT reaction was run at 42 °C for 15 minutes, followed by a denaturation step at 95 °C for 15 minutes and a cooling step at 5 °C for 5 minutes. Amplification was initially performed at 95 °C for 2 minutes and continued for 35 cycles of melting (95 °C for 1 minute) and annealing-extending with Taq thermostable polymerase (60 °C for 1 minute), followed by a final step at 72 °C for 7 minutes. PCR products were separated in a 1.5% agarose gel and semiquantitated from video images by densitometry using the Image 1.37 program (from Wayne Rasband, National Institute of Mental Health, Bethesda, MD).

Detection of Pgp by Immunoflow Cytometry Using C219 and MRK16 Antibodies

Parental nontransduced HCT15 and HCT116 cells and empty vector transduced cells, as well as TNF- or IL-2-expressing cell clones, were trypano-sinized and harvested in PBS. If the monoclonal antibody C219 (Signet Laboratories Inc.,...
Dedham, MA) was used, which detects an intracytoplasmatic epitope of Pgp. Cells were permeabilized by incubation in 3.7% formaldehyde for 10 minutes at room temperature and washed once with PBS. Cells were then resuspended in 2% heat-inactivated human AB serum (Irvine Scientific Co., Inc., Santa Ana, CA) for 5 minutes at room temperature to block nonspecific antibody binding. Cells were washed and then incubated with either C219 or MRK16 in PBS solution containing 2% bovine serum albumin and 2 µg of C219 per 5 × 10^6 cells for 60 minutes, or in a 1:100 dilution of MRK16 (Hoechst Japan Ltd., Kawagoe, Japan) for 30 minutes. Cells that were incubated with mouse immunoglobulin G (IgG1) (Becton Dickinson Immunocytometry Systems, San Jose, CA) served as negative controls. As a secondary antibody, fluorescein-conjugated goat-antimouse antibody (TAGO, Inc., Burlington, CA) was used to treat cells for 30 minutes at 4 °C. Cells were washed, and fluorescence intensity of 1 × 10^6 cells per measurement was determined with a FACScan flow cytometer (Becton Dickinson). Quantitative analysis was carried out with the LYSYS software program.

**Uptake of the Fluorescent Drug Doxorubicin**

To estimate the functional activity of Pgp, the uptake of the fluorescent multidrug resistance-related drug doxorubicin (Sigma) was determined by flow cytometry. For this analysis, cells were cultured in phenol red-free RPMI-1640 medium supplemented with 10% FCS for 48 hours and were then trypsinized and washed with phenol red-free RPMI 1640–5% FCS. Cells were aliquoted and were incubated at 37 °C in phenol red-free RPMI 1640–5% FCS containing 50 µM doxorubicin for 3 hours. Thereafter, cells were washed twice with medium and kept on ice. The fluorescence intensity of 1 × 10^5 control cells and TNF- or IL-2-expressing cell clones was determined by flow cytometry and analyzed with the use of the LYSYS software program.

**Chemosensitivity Assay**

The influence of cytokine gene transfer and expression on chemosensitivity of human tumor cells was investigated using the XTT—i.e., [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino)carbonyl-2H-tetrazolium hydroxide]—colorimetric cytotoxicity assay (23). Cells (1 × 10^3) were plated into each well of 96-well microtiter plates (Corning Corp., Costar, Cambridge, MA) and grown for 24 hours. The medium was then removed, and 200-µL dilutions of the appropriate drug were added (doxorubicin, 50-2000 ng/mL; vincristine, 50-150 ng/mL; Sigma); cells were incubated for 3 days at 37 °C. After incubation medium was removed, 50 µL XTT solution (1 mg/mL XTT in serum-free medium), and 0.02 mM N-methylphenazonium methosulfate per well was added and the cells were incubated for 4 hours at 37 °C. Absorbance was determined at 450 nm in a microplate reader. The absorbance of untreated control groups was taken as 100% survival, and the percentage inhibition was calculated as follows:

\[
\text{Growth inhibition} (\%) = 100 - \frac{100 \times (T - B)}{(U - B)}
\]

where \(T\) = treated (absorbance determined when tumor cells are exposed to drugs), \(U\) = untreated (absorbance of untreated cells), and \(B\) = blank (absorbance when neither the drug nor XTT was added).

**Statistical Analysis**

In all studies, levels of statistical significance were evaluated with data from at least three independent experiments. For statistical analysis of the flow cytometry experiments (Pgp detection as well as drug uptake assays), the nonparametric Mann–Whitney rank sum test was used. The statistical significance of the chemosensitivity assay (XTT cytotoxicity assay) was evaluated using parametric Student’s t test. Statistical significance was set at the .05 α value, and all P values reported were derived from two-sided statistical tests.

**Results**

**Transfection of pM3CMV-hTNF and pM3CMV-hIL2 Retroviral Expression Vectors and Establishment of Cell Clones**

After electroporation of cells with pM3CMV-hTNF or pM3CMV-hIL2 retroviral vector constructs, clones were isolated and expanded for further studies. Total cellular RNAs were isolated from selected cell clones of both cell lines, and supernatants were harvested to determine TNF or IL-2 expression and cytokine secretion. RT–PCR analysis using TNF- and IL-2-specific primers revealed that the selected cell clones of both cell lines expressed specific mRNAs for either TNF (702 bp) or IL-2 (462 bp). This is illustrated in Figs. 1 and 2. Neither non-transduced parental cells nor the clones transduced with the empty vector pM3neo produced TNF- or IL-2–specific PCR products. Thus, endogeneous production of the two cytokines was not detectable in the respective control cells, indicating that HCT15 and HCT116 colon carcinoma cells do not naturally express TNF and IL-2 mRNA. The expression levels of cytokine-specific mRNAs in the transfected cell clones corresponded well to the levels of biologically active cytokine proteins released.
Table 1. Secretion of human tumor necrosis factor-α (TNF) and interleukin 2 (IL-2) by transduced human HCT15 and HCT116 colon carcinoma cell clones*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clones</th>
<th>Human TNF, pg/mL</th>
<th>Human IL-2, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT15</td>
<td>pM3neo</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hTNF-1</td>
<td>1550</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hTNF-3</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hTNF-4</td>
<td>450</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hIL2-1</td>
<td>0</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hIL2-2</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hIL2-4</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>HCT116</td>
<td>pM3neo</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hTNF-2</td>
<td>450</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hTNF-4</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hTNF-5</td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hIL2-1</td>
<td>0</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hIL2-2</td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>pM3CMV-hIL2-5</td>
<td>0</td>
<td>550</td>
</tr>
</tbody>
</table>

*Cytokine amounts were determined in duplicate using TNF- or IL-2-specific enzyme-linked immunosorbent assays (ELISAs) and represent the amounts that were secreted by 1 x 10^6 cells in 24 hours into the cell culture medium. Values represent the means of duplicate determinations from three independent experiments. hTNF = human tumor necrosis factor-α; hIL2 = human interleukin 2.

Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mdr1, tumor necrosis factor-α (TNFα), interleukin 2 (IL-2), and β-actin messenger RNA expression in cytokine gene-transduced HCT116 colon carcinoma cells. Lane 1 = DNA molecular weight marker VI (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany); lane 2 = parental non-transduced cells; lane 3 = pM3neo transduced cells; lane 4 = clone pM3CMV-hTNF-2; lane 5 = clone pM3CMV-hTNF-4; lane 6 = clone pM3CMV-hTNF-5; lane 7 = clone pM3CMV-hIL2-1; lane 8 = clone pM3CMV-hIL2-2; and lane 9 = clone pM3CMV-hIL2-5. Sizes for the specific RT-PCR products are 702 base pair (bp) for TNF, 462 bp for IL-2, 167 bp for mdr1, and 316 bp for β-actin.

It is notable for all of these mdr1 expression studies in cytokine gene-transduced tumor cell clones that the extent of the decrease of mdr1 mRNA is associated with the expression level of either TNF or IL-2. In this context, it was found that, for example, HCT15 clone pM3CMV-hTNF-1 produced the highest amount of TNF (1550 pg/mL) and showed the highest degree of mdr1 mRNA reduction for the HCT15 clones. This observation was also made for the HCT116 clone pM3CMV-hTNF-5 that produced a relatively high TNF concentration (500 pg/mL) compared with the other pM3CMV-hTNF-transduced HCT116 clones, and which was associated with the greatest decrease in mdr1 expression for this cell line. This effect was also seen in the IL-2 gene-transduced colon carcinoma cell clones of both lines, so that those clones with the highest IL-2 expression level also developed the greatest extent of decrease of mdr1 mRNA.

Analysis of Pgp Expression in Cytokine Gene-Transduced Cells

To further evaluate cytokine-mediated modulation of mdr1 expression, transduced cells of both lines were labeled with the Pgp-specific antibodies MRK16 or C219, incubated with a
fluorescein-conjugated secondary antibody, and analyzed by flow cytometry. The mean fluorescence values were compared with those obtained from nontransduced parental or empty vector-harboring control cells.

In nontransduced HCT15 control cells, the mean fluorescence was 135.0 and for nontransduced HCT116 controls was 43.0 when the MRK16 antibody was used for cell labeling. When the cells were labeled with the C219 antibody, the mean fluorescence for nontransduced HCT15 controls was 67.0 and for nontransduced HCT116 controls was 52.5. Consistent with previous studies, these mean fluorescence values demonstrate a much higher intrinsic Pgp expression in HCT15 than in HCT116 control cells.

Figs. 3 and 4 show the cytokine-mediated alterations in Pgp expression in clones of both cell lines as determined with either MRK16 or C219. As illustrated in Fig. 3 for MRK16-labeled cells, transduction of TNF- or IL-2-reduced Pgp expression in HCT15 and HCT116 clones, a statistically significant reduction in Pgp expression compared with the nontransduced and empty vector-harboring control cells. This reduction in Pgp expression ranged from 1.6- to 4.3-fold in TNF-producing HCT15 clones (P < 0.0001) and from 1.3- to 1.9-fold in IL-2-expressing HCT15 clones (P < 0.0001). Similar modulatory effects in Pgp expression were seen in cytokine gene-transduced HCT116 cells, which ranged between 1.5- to 2.1-fold in the TNF-secreting clones (P = 0.0243) and from 1.38- to 1.57-fold in IL-2-expressing clones (P = 0.0243).

These observations with MRK16-labeled cells were confirmed by flow cytometry measurements of C219-labeled cells (Fig. 4), again demonstrating cytokine-mediated modulation of Pgp expression. The results obtained with two antibodies by flow cytometry are in agreement with the results of mdr1 expression analysis by RT-PCR of transduced HCT15 and HCT116 cells. These results indicate that reduction of mdr1 expression by TNF or IL-2 leads to significantly reduced Pgp expression (P < 0.0087). Of interest, cytokine concentration dependence of the extent of the decrease on the Pgp level could also be seen in the FACScan analysis, supporting the findings.

---

**Fig. 3.** P-glycoprotein (Pgp) expression in cytokine gene-transduced human colon carcinoma cells detected with the monoclonal anti-Pgp-antibody MRK16. A = HCT15; B = HCT116. Fluorescence intensity was measured by the use of a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer and expressed as mean fluorescence (obtained in three independent experiments). Statistical significance was evaluated using the two-sided Mann–Whitney rank sum test. hTNF = human tumor necrosis factor-α; hIL2 = human interleukin 2.

**Fig. 4.** P-glycoprotein (Pgp) expression in cytokine gene-transduced human colon carcinoma cells detected with the monoclonal anti-Pgp-antibody C219. A = HCT15; B = HCT116. Fluorescence intensity was measured by the use of a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer and expressed as mean fluorescence (obtained in three independent experiments). Statistical significance was evaluated using the two-sided Mann–Whitney rank sum test. hTNF = human tumor necrosis factor-α; hIL2 = human interleukin 2.
on the mRNA level. This observation suggests that the higher the expression levels of the appropriate cytokine, the more dramatic is the decrease in Pgp expression in these cells.

**Doxorubicin Uptake in Transduced HCT15 and HCT116 Cells**

To examine the functional importance of the effects of the transduced TNF or IL-2 gene on mdr1 and Pgp expression, accumulation of the multidrug resistance-related drug doxorubicin was determined by FACScan analysis exploiting the fluorescent properties of this compound. Fig. 5 summarizes the data determined as the mean fluorescence values per cell after 3 hours of doxorubicin incubation in control and cytokine gene-transduced cells. The doxorubicin uptake in nontransduced parental and empty vector-transduced control cells was 30.0 for HCT15 and 151.5 for HCT116 cells. This result further characterizes the differences in multidrug resistance phenotype on the functional level between the two lines, since the uptake in more sensitive HCT116 cells was five times higher than in multidrug-resistant HCT15 cells. As shown in Fig. 5, transduction of either TNF or IL-2 dramatically enhances doxorubicin accumulation in HCT15 and HCT116 cell clones, as reflected by the higher values of mean fluorescence compared with the appropriate control cells. This enhancement in drug accumulation was 2.16-fold to 2.6-fold higher in TNF-expressing HCT15 clones \((P<.0001)\) and 1.8-fold to 2.7-fold higher in IL-2-expressing HCT15 clones \((P<.0001)\).

Doxorubicin uptake was enhanced 1.4-fold to 1.8-fold in TNF-expressing HCT116 clones \((P<.0001)\) and increased 1.3- to 1.4-fold in IL-2-expressing HCT116 clones \((P<.0001)\). In both tumor cell lines, the highest drug fluorescence was measured in those cell clones that also produced the highest amounts of either TNF or IL-2. This supports the previous findings of cytokine-mediated modulation of mdr1 and Pgp expression in these clones and confirms the cytokine concentration dependence of the multidrug resistance modulatory effects.

**Chemosensitization of Transduced Cells**

To examine potential alterations in chemosensitivity, the multidrug resistance-associated drugs vincristine and doxorubicin were tested on nontransduced parental and empty vector-harboring control cells and on transduced clones of both cell lines. Treatment was carried out over a concentration range of 50-1500 ng/mL with vincristine or doxorubicin for 3 days. Cytotoxic effects were expressed as a percentage of growth inhibition compared with the respective untreated control cells. In separate experiments, the influence of secreted TNF or IL-2 on cell proliferation of transduced HCT15 and HCT116 clones was determined by comparison to nontransduced and empty vector-transduced cells. These experiments revealed that the cytokine expression did not influence the growth behavior of the clones. The cytotoxic activity of vincristine and doxorubicin increased for transduced clones of both cell lines (Fig. 6, A-D and Fig. 7, A-D). However, although both anticancer drugs were observed to be more effective in transduced than in control cells, it was notable that vincristine activity was enhanced more compared with doxorubicin. Treatment with vincristine led to 80%-100% growth inhibition in TNF- or IL-2-transduced clones of both cell lines (Fig. 6, A \([P<.035]\), C \([P<.038]\); Fig. 7, A \([P<.0054]\), C \([P<.046]\)), whereas the maximum growth inhibition after doxorubicin treatment ranged between 60% and 80% in these clones (Fig. 6, B \([P<.038]\), D \([P<.033]\); Fig. 7, B \([P<.032]\), D \([P<.046]\)). Besides this, chemosensitization caused by the cytokine gene transfer was dramatic in both cell lines for both anticancer drugs. This was reflected by the fivefold increase in vincristine cytotoxicity for TNF- or IL-2-expressing HCT15 clones and a threefold to fourfold increase in doxorubicin cytotoxicity for these cytokine-expressing HCT15 clones. In TNF- or IL-2-expressing HCT116 clones, vincristine cytotoxicity was increased 1.8-fold to 2-fold and 1.8-fold to 2.3-fold for doxorubicin.

The influence of cytokine concentration on the cytotoxic effects of cytokine-drug combination is shown best in Fig. 6, A and B for HCT15 clone pm3CMV-hTNF-1 that releases the highest TNF concentrations. This clone demonstrates the highest degree of chemosensitization to vincristine and doxorubicin, as reflected in the fourfold to fivefold increase in cytotoxicity of these drugs.
Fig. 6. XTT—(i.e., [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl-2H-tetrazolium hydroxide])—cytotoxicity assay in doxorubicin- or vincristine-treated cytokine gene-transduced HCT15 cells. A = cytotoxicity of vincristine in pM3CMV-hTNF-transduced cells; B = cytotoxicity of doxorubicin in pM3CMV-hTNF-transduced cells; C = cytotoxicity of vincristine in pM3CMV-hIL2-transduced cells; and D = cytotoxicity of doxorubicin in pM3CMV-hIL2-transduced cells. Tumor cells were transduced with pM3CMV-hTNF or pM3CMV-hIL2, respectively, as described in the “Materials and Methods” section. Cells were then incubated with doxorubicin or vincristine at indicated concentrations for 3 days. Cytotoxicity was determined in triplicate by XTT colorimetric assay in three independent experiments and expressed as percent growth inhibition. Variations (standard deviation) were less than 15% of the total measurements. Statistical significance was evaluated using a two-sided Student’s t-test. Nontransduced parental cells (○), clone pM3neo (△), clone pM3CMV-hTNF-1 (●), clone pM3CMV-hTNF-3 (◇), clone pM3CMV-hTNF-4 (+), clone pM3CMV-hIL2-1 (●), clone pM3CMV-hIL2-2 (◇), and clone pM3CMV-hIL2-4 (+). CMV = cytomegalovirus; hTNF = human tumor necrosis factor-α; hIL2 = human interleukin 2.

Discussion

Multidrug resistance appears to be one of the major obstacles to successful chemotherapeutic treatment of cancer. This has led to a great research effort to find approaches to reverse the multidrug resistance phenotype in resistant tumors. It has been established for a considerable time that the combination of certain cytokines and drugs can produce additive or synergistic antitumor effects. This has been shown for the combination of TNF and etoposide or doxorubicin, and also with cisplatin, fluorouracil, or mitoxantrone (24-27). Potentiating effects have also been reported for IL-2 combined with doxorubicin, vincristine, dacarbazine, cisplatin, or fluorouracil (28). In addition to these studies, other investigators (5,6) have demonstrated that external application of cytokines, including TNF and IL-2, can modulate mdr1 expression and improve efficacy of multidrug resistance-related drugs. However, the severe side effects of systemic application of either TNF or IL-2, such as fever, rigors, myalgias, capillary leak syndrome, etc., limit their clinical application. For this reason, the antitumor activity of cytokines, such as TNF and IL-2, might be optimally exploited if applied in the tumor vicinity by intratumoral or locoregional treatments (29-31). The direct delivery of cytokines to the tumor cells by transfer of cytokine genes has previously been shown to overcome systemic toxic effects in vivo and to provide for antitumor immune responses (32-35). Thus, cytokine gene transfer has become an important strategy in human gene therapy trials that are using TNF- or IL-2-secreting tumor cells for cancer treatment (36,37).

In the present study, the therapeutic potential of a combination of gene therapy and chemotherapy on the basis of cytokine-
mediated modulation of multidrug resistance in human colon carcinoma cells was investigated. The effects of TNF or IL-2 gene transfer were analyzed in detail on the mdr1 mRNA and Pgp expression levels and on the functional level by measuring drug accumulation. The influence of cytokine gene transfer on multidrug resistance phenotypes in HCT15 and HCT116 cells was further evaluated by determining the cytotoxic effects of the multidrug resistance-related drugs doxorubicin and vincristine in transduced cell clones. The studies with TNF- or IL-2-expressing cell clones revealed that cytokine gene transfer reduces mdr1 mRNA expression, which correlates well with the cytokine-mediated effects on Pgp expression. Flow cytometry measurements of tumor cell clones of both cell lines also showed a dramatic decrease in Pgp expression in either MRK16- or C219-labeled cells. As a result of the decrease of mdr1 mRNA and of Pgp in cytokine gene-transduced cells, alterations in Pgp function could be expected. This was indeed measurable in the TNF- and IL-2-expressing clones. The uptake of the fluorescent drug doxorubicin was elevated in these cell clones, providing direct evidence of increased drug accumulation within the cells. To further demonstrate the functional importance of these cytokine-mediated activities on multidrug resistance, the cytotoxic effects of the multidrug resistance-related drugs doxorubicin and vincristine were measured by XTT cytotoxicity assay. These experiments demonstrated a close relationship between the reduction of mdr1 and Pgp expression and the enhanced cytotoxic effects of the two drugs in all TNF- and IL-2-expressing clones of both cell lines. In addition, the cytokine-mediated effect of multidrug resistance modulation was shown to be cytokine concentration dependent, so that higher cytokine amounts caused more dramatic effects on multidrug resistance reversal.
These findings support and extend the results of our earlier studies, where external application of cytokines, including TNF and IL-2, was found to reverse the multidrug resistance phenotype in human colon carcinoma cells (6). More recently (14), we have reported that gene transfer of the human TNF gene into human glioblastoma cells caused both reduction of mdrl and Pgp expression and sensitization of transduced cells to doxorubicin and vincristine. In addition, earlier observations in fluorouracil-treated TNF-transduced colon and mammary carcinoma cells corroborates the specificity of cytokine-mediated effects on multidrug resistance: although TNF expression could slightly enhance fluorouracil cytotoxicity in these cells, this effect did not reach the level of cytotoxicity that has been accomplished by the combination of TNF- and multidrug resistance-associated drugs.

The present data on TNF- or IL-2-gene-transduced HCT15 and HCT116 cells provide a more detailed line of evidence that cytokines of different natures can act as multidrug resistance-reversing agents in tumor cell lines with different levels of intrinsic multidrug resistance. Furthermore, the present study demonstrates that this phenomenon is not limited to TNF gene transfer and expression, since similar results were obtained with IL-2.

As mentioned previously, it has been reported in several studies (38,39) that externally applied cytokines can modulate the multidrug resistance phenotype, sensitizing tumor cells to multidrug resistance-related drugs or preventing resistance acquisition in cytokine-treated cells. However, in only a small number of reports was cytokine-mediated modulation of mdrl mRNA and Pgp expression described (5,6,27,40-42). This could be explained in part by the finding that cytokine-mediated multidrug resistance modulatory effects seem to be strictly time dependent, requiring long-term treatments (48-72 hours) to induce a decrease in mdrl expression. This has been demonstrated for external TNF, IL-2, and interferon alfa application (6). It is possible that transduction of cytokine genes and subsequent release of cytokines by transduced tumor cells might mimic the long-term external cytokine treatment, leading to effective multidrug resistance reversal in these cells.

This article represents one of the first descriptions of cytokine-mediated multidrug resistance reversal in transfectected tumor cell clones that directly link cytokine gene transduction and expression and the multidrug resistance phenotype in these cells. Combination of gene therapy and chemotherapy may represent an important new strategy in treatment of cancers. The present approach consists of two components: multidrug resistance-reversing cytokine gene transfer and the subsequent chemotherapeutic treatment of cytokine-releasing tumor cells at lower, but still effective, doses of multidrug resistance-associated drugs to kill these cancer cells. However, more preclinical research is required to fully develop such a combined treatment approach. For example, further in vitro and in vivo studies are needed to establish whether cytokine gene-transduced tumor cells can also influence neighboring nontransduced cancer cells to render them more susceptible to cancer chemotherapy. Application of this combined modality strategy to in vivo studies requires development of methods that ensure efficient gene delivery to the target tumor cell, since gene delivery in vivo must be accurate and must result in appropriate gene expression. Future investigations will be directed toward identifying a gene delivery protocol that can transduce enough target cells in vivo to achieve a sufficient effect on multidrug resistance modulation in this context of combined gene therapy and chemotherapy. The present study has clearly shown that the strategy of multidrug resistance reversal by cytokine gene transfer can work in an experimental setting, and it is therefore reasonable to pursue this approach for potential clinical applications in tumor therapy.

References


Notes

Supported by Feodor-Lynen fellowships (U. Stein, W. Walther) from the Alexander von Humboldt Foundation, Bonn, Federal Republic of Germany, and by the Office of International Affairs, National Cancer Institute.

Manuscript received February 1, 1996; revised June 10, 1996; accepted July 5, 1996.