Doxycycline potentiates antitumor effect of cyclophosphamide in mice

Rishi Raj Chhipa, Sandeep Singh, Sachin V. Surve, Maleppiliil Vavachan Vijayakumar, Manoj Kumar Bhat*

National Centre for Cell Science, Pune University Campus Ganeckhind, Pune, Maharashtra, 411 007, India

Received 1 April 2004; accepted 29 June 2004

Cyclophosphamide (CPA) is a widely used chemotherapeutic drug in neoplasms. It is a DNA and protein alkylating agent that has a broad activity against variety of neoplasms including breast cancer. The therapeutic effectiveness of CPA is limited by the high-dose, renal, and cardiac toxicity that accompanies the systemic distribution of liver-derived activated drug metabolites. The present study evaluated the potential of combining well-tolerated antibiotic doxycycline (DOX) with CPA and understanding the mechanism of synergism, we found that DOX significantly enhances the tumor regression activity of CPA on xenograft mouse model bearing MCF-7 cells. DOX also potentiates MCF-7 cell killing by CPA in vitro. In presence of DOX (3 μg/ml), the IC50 value of CPA decreased from 10 to 2.5 mM. Additional analyses indicate that the tumor suppressor p53 and p53-regulated proapoptotic Bax were downregulated in vivo and in vitro following CPA treatment in combination with DOX, suggesting that upregulation of p53 may contribute to enhancement of antitumor effect of CPA by DOX. Furthermore, downregulation of antiapoptotic Bcl-2 was observed in animals treated with CPA plus DOX when compared to untreated or DOX-treated groups. Our results raise the possibility that this combination regimen may lead to additional improvements in treatment of breast cancer.

Keywords: Cyclophosphamide; Doxycycline; p53; Bax

In contrast, the therapeutic efficacy of two or more drugs may have greater beneficial effects on cancer treatment and management. CPA is a cell cycle-dependent DNA and protein alkylating agent that has a broad spectrum of activities against variety of neoplasms, and is widely used in the clinical management of human malignancies including breast cancer (Moore, 1991; Sladee, 1988). In conventional chemotherapy, CPA is one of the most commonly employed drugs and is used in high-dose regimen to treat metastatic breast cancer (Legha et al., 1979; Tormey et al., 1982). This setting the therapeutic efficacy of this drug is limited by host toxicity as a result of the systemic distribution of activated drug metabolites that have significant cytotoxic effects including cardiac and renal toxicity (Chen et al., 1996). Therefore, drugs that could potentiate CPA antineoplastic effect would be of significant importance and lessen the toxic effects associated with high-dose CP treatment. Potentiation of antitumor effect of CPA by AQ41...
and tirapazamine on mammary carcinoma (Friery et al., 2000), and by bis-indole alkaloid on breast cancer cells has been reported (Leung et al., 2000). Very recently it has been demonstrated that coadministration of thalidomide and CPA gave markedly greater activity against Colon 38 tumor compared with either drug alone (Ding et al., 2002).

Doxycycline (DOX), a commonly used antibiotic, has antitumor activity against several malignancies (Fife et al., 1998; Rubins et al., 2001). Recently it has been reported that DOX has potential treatment value in bone metastasis of breast cancer cells (Duivenvoorden et al., 2002). DOX inhibits these effects by inhibiting matrix metalloproteinases (MMPs), not only in breast cancer cells but also in human endothelial, prostate cancer, osteosarcoma cells of patients (Fife et al., 1997; Hanemannajer et al., 1998). Moreover, it also inhibits cell proliferation and induces apoptosis in various cancer cells (Rubins et al., 2001). All these studies demonstrate that this well-tolerated antibiotic may be effective in treatment of various human cancers, either alone or in combination therapy.

DOX is potentially beneficial in bone metastasis of breast cancer cells and CPA is an important component of chemotherapeutic regimen for treatment of breast cancers. Therefore, we postulated that this combination treatment might enhance antitumor effect of CPA on breast cancer cells in vivo. We found that DOX significantly enhances the tumor regression activity of CPA on xenograft mice model of MCF-7 cells. In addition, we explored the molecular basis of enhanced toxic effect of CPA in presence of DOX both in vitro and in vivo. Our results raise the possibility that this combination chemotherapeutic regimen may lead to additional improvements in treatment of breast cancer.

Material and methods

CPA, DOX, and methyl-thiazolyl tetrazolium (MTT) were purchased from Sigma, St. Louis, USA, and dissolved in sterile water to prepare a stock of 50 mM, 5 mg/ml and 1 mg/ml, respectively. Human breast cancer line MCF-7 (ATCC HTB-22) was obtained from ATCC, VA, USA and maintained in our in-house National Cell Repository. Cells were routinely cultured in MEM supplemented with 10% heat-inactivated fetal bovine serum (HyClone, UT, USA), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Invitrogen Life Technologies, Maryland, USA), at 37 °C with 5% CO2. Wherever tetracycline inducible system (Tet system) has been used, MEM was supplemented with Tet system approved fetal bovine serum (Clontech, CA, USA).

Animals, animal-keeping conditions, and tumor growth regression model

Eight weeks old female homozygous (nu+/nu+) nude athymic NIH mice (NIV, Pune, India), 18–22 g, were used as human cancer xenograft models. The animals were housed in specific pathogen-free isolators (Harlan isolotec, USA) in an environmentally controlled Animal Care facility of our institute. They were caged (six per cage) in polypropylene boxes and had free access to sterilized pelleted laboratory rodent chow and water. All animal experiments have been performed following the requirement of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and after permission of the Institute’s Animal Care and Use Committee (IACUC). Five consecutive subcutaneous (sc) estradiol valerate (30 μg/day) injections were administered in each mouse before subcutaneous injection of MCF-7 tumor cells in the exponential growth phase (2 × 106 cells in 0.1 ml) at right flank of each mouse. Drug treatments were initiated 2 weeks after palpable tumors developed. The mice were randomized into four groups having six mice per group (n = 6) to receive vehicle alone (control), DOX (5 mg/kg/day), CPA (100 mg/kg/day), or CPA plus DOX by intraperitoneal (ip) injection for four consecutive days. CPA and DOX were dissolved in PBS. Four days following last injection mice were euthanized and tumors were surgically excised. Tumor growth was determined by weight measurements and host toxicity was monitored by body weight measurements. Representative tumor tissues (200 mg from each animal of a group) were processed and samples were pooled from three animals per treatment group for reverse transcriptase polymerase chain reaction (RT-PCR) analysis as mentioned.

MTT cell proliferation assay

To assay the cell viability after drug treatment, cells were seeded at a density of 7500 cells per well into 96-well plates and allowed to adhere for 24 h at 37 °C. Cells were treated with various concentrations of CPA and CPA plus DOX (1 and 3 μg/ml) for 24 h in triplicates and further incubated for additional 48 h in absence of drugs. Thereafter, media was decanted and 50 μl of MTT (1 mg/ml) in MEM (without phenol red) was added to each well and incubated for 4 h at 37 °C. Formazan crystals were solubilized in 50 μl of isopropanol by incubating with shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm as reference filter. Absorbance given by untreated cells was taken as 100% cell growth.

RNA extraction, cDNA synthesis, and RT-PCR

Total cellular RNA from treated and untreated cells were extracted using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. Five micrograms of total RNA and oligo (dT)12–18 primers were taken in diethyl pyrocarbonate (DEPC)-treated water. cDNA synthesis was initiated using 200 units of M-MLV reverse transcriptase (Invitrogen Life Technologies) under conditions recommended by manufacturer and the reaction was allowed to proceed at 37 °C for 50 min. Reaction was
154 terminated by heating at 70 °C for 15 min. Each RT-PCR
155 contained 10% of CDNA, 20 pmol of each primer in 20 mM
156 Tris–HCl (pH 8.4) containing 50 mM KCl, 1.5 mM MgCl₂,
157 0.2 mM dNTP mix, and 1 unit of platinum Taq DNA
158 polymerase (Invitrogen Life Technologies) in a final volume
159 of 20 μl. After an initial denaturation for 1 min at 95 °C, 30
160 and 35 cycles of denaturation (94 °C for 1 min), annealing
161 (for 1 min), and extension (72 °C for 1 min) were performed
162 on a DNA thermal cycler (Techne, Cambridge, UK) with a
163 final expansion for 10 min at 72 °C. The primer pairs used
164 were as follows: p53 5'-CTG AGG TTG GCT CTG ACT
165 GTA CCA CCATCC-3' (F), 5'-CTC ATT CAG CTC TCG
166 CG-3' (R), Bax 5'-ATG GAC CG-3' (F), Bcl-2, p21, and p-actin
167 Bax, Bcl-2, p21, and p-actin PCR were 55 °C, 59 °C,
168 64 °C, respectively.
169
170 Immuno blotting
171 Following 36-h treatment with CPA in presence or
172 absence of DOX, cells were washed three times with ice-cold
173 PBS and lysed in 100 μl of lysis buffer (20 mM HEPES pH
174 7.4 containing 1% Triton X-100, 2 mM EDTA, 2 mM
175 EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM
176 PMSF, 1 mM TPC2, 2 mM NaVO₄, and protease
177 cocktail tablet (Boehringer Mannheim, Germany) per 1 ×
178 10⁶ cells on ice. An equal amount of protein samples (75 μg)
179 were resolved on 10–12% SDS–polyacrylamide gel and then
180 transferred onto PVDF membrane (Amersham, Aylesbury,
181 UK). The membranes were probed with antibodies against
182 p53, Bax, and β-actin (Santa Cruz Biotechnology, CA,
183 USA). The immuno blots were detected by enhanced
184 chemiluminescence (ECL) reagent (New England Biolabs,
185 Massachusetts, USA).

187 Transfection and chloramphenicol acetyl transferase (CAT)
188 reporter assay
189 The p53 CAT reporter construct PG₃₂CAT, which
190 contains 13 repeats of p53 binding site inserted 5' to
191 polyomavirus basal promoter linked to CAT reporter gene
192 (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore,
193 USA) along with a β-galactosidase expression vector
194 (pCH101), was transiently transfected MCF-7 cells by
195 lipofection method (Invitrogen Life Technologies). After
196 transfection, cells were washed and fresh media with or
197 without drugs was added to the culture plates for an
198 additional 24 h. Cells were then collected and washed
199 thrice with PBS and resuspended in 0.25 M Tris–Cl (pH 7.5)
 buffer. Cells were lysed by four cycles of rapid freeze–thaw.

200 CAT assay was performed by taking equal amounts of lysate
201 protein in presence of 1 μCi C₁₄-chloramphenicol (NEC,
202 Boston, MA, USA) and 100 μg of acetyl CoA (Amersham)
203 in 250 mM Tris–Cl (pH 7.5) in a total reaction volume of
204 100 μl. Reaction mixture was incubated at 37 °C for 5 h
205 and terminated by adding ethyl acetate to the sample tubes.
206 Products were resolved by thin layer chromatography
207 (TLC), using mixture of chloroform and methanol (19:1
208 ratio). TLC plates were analyzed by autoradiography and
209 scanning on a phosphorimager (Bio Rad, California, USA).

210 Transient expression of antisense p53 in MCF-7 cells
211 p53-dependent cell death in MCF-7 cells treated with
drug combinations was assessed by transiently co-transfecting
cells with pTet-ON (Clontech) and pTRErevp53
(vector for antisense p53 expression). In pTRErevp53
plasmid, p53 was subcloned for antisense orientation in
parental pTRE vector (Clontech), which has tetracycline
responsive element (TRE) specifically responsive to
minimal doses of DOX. Briefly, 1800 bp fragment of
wild-type p53 was excised with BamH1 (New England
Biolabs) restriction digestion from pCS3-SN3 vector (kind
gift of Dr. Bert Vogelstein, John Hopkins, Baltimore,
USA) and subcloned in antisense orientation in multiple
cloning sites of pTRE vector. The correctness of pTRErevp53
was confirmed by sequencing as well as by partial
restriction mapping (data not shown). Moreover, the
functionality of antisense p53 was also proved by
evaluating its effect on p53 transactivation activity (data
not shown). This system has got inherent advantage as it
utilizes DOX as an inducer. Briefly, cells were plated in
six-well plate and transfection was performed by lipofecto-
amine method (Invitrogen Life Technologies). After trans-
fection, cells were washed and fresh media with or without
drugs was added to the cell in culture plates for an
additional 48 h. Cells were then harvested for PI staining
as well as for immuno blotting. Transfection efficiency
was determined simultaneously by transfecting green fluores-
cent protein expressing plasmid pEGFPN1 (Clontech). It
was also used as an internal control for normalization of
transfection efficiencies.

215 Flow cytometry for cell cycle analysis
216 Cells were plated at a density of approximately 8 × 10⁵
217 cells in 60-mm tissue culture plates and allowed to grow for
218 24 h. Ten millimolar CPA and 3 μg/ml DOX were added to
219 the cells for 48 h. Cells were harvested by trypsinization and
220 subsequently processed for flow cytometric analysis. In
221 brief, cells were washed twice in chilled PBS and fixed in
222
70% ethanol on ice. After RNase A (200 μg/ml) treatment for 30 min at 37 °C, 50 μg/ml propidium iodide (PI) was added to cell pellet and incubated in the dark for 30 min on ice. The fluorescence of PI was collected through a 585-nm filter in FACScan flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). The data were analyzed using the Cell Quest Software, for 10,000 cells.

Total cell death determination by flow cytometric analysis

For the determination of cell death, MCF-7 cells were treated with various concentrations of DOX (0.3 to 6 μg/ml) in presence of CPA (10 mM) as described earlier. The cells were stained with propidium iodide (50 μg/ml) and analyzed by the method of Nicoletti et al., 1991 and Wolbers et al., 2004. This method offers advantage for being quantitative for calculation of cell death indices. The fluorescence of PI was collected through a 585-nm filter in FACScan flow cytometer (Becton Dickinson GmbH). Both dot plot and histogram analyses were performed using the Cell Quest Software (Becton Dickinson GmbH, Heidelberg, Germany), for 10,000 cells.

Results

Effect of doxycycline on the antitumor activity of cyclophosphamide

CPA, administered at 100 mg/kg/day in nude mice bearing tumor derived by injecting MCF-7 cells resulted in decreased tumor size by 50% compared to tumors in untreated animals (Fig. 1B). DOX injection alone did not significantly induce tumor regression. The co-administration of DOX (5 mg/kg/day) along with CPA further enhanced the tumor regression by more than 25% (Figs. 1B and C). Therefore, it is clear that DOX, one of the better-absorbed antibiotics with longer half-life, can potentiate the antitumor activity of CPA in vivo. To ascertain genes that may be involved in enhanced tumor regression following CPA and DOX treatment, RT-PCR assay was carried out to monitor expression pattern of major cell growth regulatory genes. CPA treatment lead to increase in pro-apoptotic Bax by 1.2-fold that was further enhanced to 1.4-fold by DOX treatment. Concurrently, the level of antipapoptotic gene Bcl-2 decreased significantly in CPA and CPA plus DOX-treated tumors (Fig. 1D). Moreover, DOX treatment also enhances p53 transcript level without alteration in p21 gene transcript. These results suggest that slight but reproducible enhancement of p53 and Bax transcript levels may be responsible for DOX-mediated potentiation of CPA-induced tumor regression. To further investigate the DOX enhanced tumor regression property of CPA, in vitro studies on MCF-7 cells were performed.

MTT cytotoxicity assay

A dose-response curve of the MCF-7 cells with respect to various concentrations of CPA and DOX was generated. As shown in Fig. 2A, the treatment of 10 mM concentration of CPA alone, about 55% cells were viable compared to untreated cells. DOX alone up to a concentration of 7 μg/ml did not affect the viability of MCF-7 cells (Fig. 2A, inset). Experiments were then performed to determine if DOX and CPA together enhance the cytotoxicity. Interestingly, it appears that treating cells with varying concentrations of CPA in presence of DOX (0.3 to 6 μg/ml) increased the cytotoxicity of CPA. The treatment of 10 mM CPA in presence of 3 μg/ml DOX led to 25% cells were viable (Fig. 2A). The IC50 value of CPA dropped to 6 mM in presence of 1 μg/ml DOX (data not shown) whereas in the presence of 3 μg/ml DOX the IC50 value further dropped to 2.5 mM (Fig. 2A). These data clearly demonstrate the enhanced cytotoxic potential of CPA on MCF-7 cells in presence of DOX.

Flow cytometric analysis for apoptosis in drug-treated MCF-7 cells

To examine the mechanism of cell death, cells treated with drugs were stained with propidium iodide and apoptotic or sub G0/G1 population was analyzed by flow cytometry. Treatment with DOX alone did not further enhance the apoptotic cell population, which further indicates that DOX (3 μg/ml) did not have any cytotoxic effect on MCF-7 cells. MCF-7 cells treated with CPA resulted in apoptotic population of 16%. Interestingly, when cells were treated with both DOX and CPA together, the apoptotic population dramatically increased to 27% (Table 1). These results indicate that CPA-induced apoptosis in MCF-7 cells is enhanced in presence of DOX.

p53 and Bax are upregulated following treatment of MCF-7 cells in vitro with a combination of DOX and CPA

The DNA-damaging chemotherapeutic drugs are known to trigger nuclear accumulation of the tumor suppressor protein p53 resulting in apoptotic cell death. We examined whether p53 is involved in apoptotic cell death induced by CPA alone or by CPA and DOX together. The MCF-7 cells, which contain functional wild-type p53, were co-transfected with a reporter construct containing p53 response element (PG13-CAT) along with a control p-galactosidase reporter plasmid (pCH110). As shown in Figs. 2B and C, p53 promoter is activated at least 3-fold (P < 0.05) in cells treated with CPA compared to untreated cells. Addition of DOX further enhanced the CPA-mediated increase in p53 reporter activity to 3-fold (P < 0.05). Thus, DOX addition enhanced the CPA-induced p53 reporter activity by at least 1.5-fold (Fig. 2C, inset). We also tested whether CPA or CPA plus DOX treatment activates expression of β-
Fig. 1. Tumor growth regression activity of cyclophosphamide is potentiated by doxycycline on human breast cancer cells (MCF-7) implanted in athymic mice. Experimental design and reference time frame (A). Tumors surgically excised from the mice injected with carrier, CPA, and CPA plus DOX (B). The tumors excised from the mice were weighed. Values are expressed as means (standard deviation, n = 6). Values between tumors from CPA and CPA plus DOX injected animals are significantly different (P < 0.01) (C). RT-PCR was performed for p53, p21, Bax, and Bcl-2 with specific gene primers listed in Materials and methods using total RNA isolated from treated and untreated MCF-7 xenograft tumors. Expression of β-actin mRNA was used as control for RNA integrity and normalization. Expression levels were measured by phosphomager densitometry scanner analysis (Bio-Rad) and relative levels are given (D).

Fig. 2D. p53 mRNA level was about 1.2- and 1.4-fold higher in cells treated with CPA and CPA plus DOX combination, respectively, compared to p53 mRNA level of untreated or DOX-treated cells. Moreover, the increase in p53 transcript level also led to increase in p53 protein by 1.6- and 2.4-fold in cells treated with CPA and CPA plus DOX combination, respectively (Fig. 2E). It is therefore likely that increase in p53 promoter activity is indeed due to increase in p53 transcript levels as well as protein level. p53 can upregulate Bax, p21, and downregulate Bcl-2.
Fig. 2. In vitro MCF-7 cell killing and p53 upregulation. MTT assay was performed as documented in Materials and methods. MCF-7 cells were incubated in presence of CPA or CPA plus DOX for 24 h, and cell survival was determined by MTT metabolism. Cell survival (mean ± SD, n = 5) is expressed as a fraction of untreated control cells (A). Inset in A demonstrates DOX effect on MCF-7. MCF-7 cells were transiently transfected with PG<sub>p53CAT</sub> reporter and treated with either carrier, CPA (10 mM) and CPA plus DOX (3 μg/ml) for 24 h. Cells were harvested for CAT assays as described in Materials and methods (B and C). Inset in C is fold CAT induction. Using total RNA isolated from MCF-7 cells treated with carrier, DOX, CPA, or CPA plus DOX, RT-PCR was performed with specific gene primers (D). MCF-7 cell lysates were electrophoresed, transferred, and immunoblotted with antihuman p53 antibody. Same blot was stripped and reprobed with antihuman β-actin antibody as an internal control (E). Expression levels were measured by phosphorimager densitometry scanner analysis (Bio-Rad). Relative levels values are normalized with β-actin. Independent experiments were repeated at least three times.
Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Apoptotic population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.69 ± 0.05</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>5.2 ± 0.35</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>16.55 ± 0.65</td>
</tr>
<tr>
<td>Cyclophosphamide + Doxycycline</td>
<td>27.30 ± 3.6</td>
</tr>
</tbody>
</table>

1.3 Values are expressed as means (standard deviation, n = 4).

381 Miyashita and Reed, 1995; Thornborrow and Manfredi, 1999). Therefore, we investigated the effect of CPA and CPA plus DOX on constitutive levels of Bax and Bcl-2 by RT-PCR and Western blot analysis. Bax transcript levels increased 1.3- and 1.5-fold (Fig. 3A), whereas the protein increased by 1.45- and 1.75-fold (Fig. 3B), in CPA and CPA plus DOX-treated cells compared to untreated control cells.

388 No change in Bcl-2 transcript level was detected. Interestingly, drug treatments downregulated Bcl-2 gene in vivo, whereas no significant changes in Bcl-2 were detected in vitro.

392 Enhanced cell death in CPA-treated MCF-7 cells is DOX dose dependent.

394 To further examine whether DOX enhances CPA-mediated cell killing in a dose-dependent manner, MCF-7 cells were treated with CPA alone or in presence of DOX (0.3 to 6 µg/ml) for 48 h and percentage of PI stained cells were analyzed by flow cytometry as described in Materials and methods. DOX at a concentration of 0.3 µg/ml did not further enhance the CPA-mediated cell killing. The percentage of cell killing by DOX (0.3 µg/ml) and CPA combination was 47.5 ± 4 compared to that of CPA alone (46.75 ± 3). Increase in DOX concentration to 1, 3, and 6 mg/ml lead to enhancement of CPA-mediated cell killing approximately by 12%, 23%, and 27%, respectively (Fig. 4A). These results indicate that DOX in a dose-dependent manner enhances CPA-mediated killing of MCF-7 cell with maximum death being achieved with DOX concentration between 3 and 6 µg/ml. Under identical experimental conditions, DOX alone did not significantly increase MCF-7 cell killing.

396 Downregulation of p53 prevents DOX enhanced MCF-7 cell killing by CPA.

398 To examine DOX-enhanced MCF-7 cell killing by CPA is indeed p53 dependent, we evaluated whether cell death induced by CPA and DOX together involved p53. MCF-7 cells were transiently transfected with either pTet-ON or pTRErevp53 plasmids alone and also cells were transiently co-transfected with pTet-ON and pTRErevp53 plasmids together. The transiently transfected cells were treated with combination of CPA and DOX for 48 h. Subsequently, cells were stained with PI and analyzed for cell death. As shown in Fig. 4B, DOX at 0.3 µg/ml did not effect cell killing by CPA. Increase in DOX to 3 µg/ml enhanced CPA-mediated cell killing. Moreover, transfection with pTet-ON or pTRErevp53 plasmids did not in anyway alters the effect of drugs on MCF-7 cells. However, MCF-7 cells in which pTet-ON and pTRErevp53 plasmids were co-transfected. DOX addition failed to enhance CPA-mediated cell killing.

399 Cell death in these cells was comparable to CPA alone treated MCF-7 cells. These results suggest that p53 is involved in enhanced cell killing effect of CPA and DOX combination. To further verify the status of p53 protein in the cells, which were transiently transfected and subsequently treated with drugs, whole cell lysate from these cells were analyzed by Western blot analysis for p53 expression. As shown in Fig. 4C, p53 protein levels are significantly higher in CPA and DOX (3 µg/ml)-treated MCF-7 cells compared to the cells which were treated with CPA and low noneffective dose of DOX (0.3 µg/ml). p53 protein levels were low in the cells in which antisense p53 was transiently transfected.
Fig. 4. PI staining of drug-treated MCF-7 cell and involvement of p53. After dose-dependent treatment of MCF-7 cells with DOX alone with CPA (10 mM) for 48 h, cells were stained with 50 μg/ml PI and analyzed in a flow cytometer for total PI-positive cells with acquisition of 10,000 cells (n = 4). The representative percentage of cell death is indicated by bar graph (A). Total cell death in MCF-7 cells, which were transiently transfected with antisense p53, followed by drug treatment (B). Cell lysate prepared from treated cells were electrophoresed, transferred, and immunoblotted with anti-human p53 antibody. Same blot was stripped and reprobed with anti-human actin antibody as an internal control (C).

145 expressed and subsequently exposed to CPA and DOX
146 (3 μg/ml) together. As a control for Western blot analysis,
147 same blot was stripped and reprobed for expression of actin
148 and no significant difference were detected. Therefore, the
149 p53 protein level in cells treated with drug combination is
150 reduced because expression of antisense p53 were detected. These results
151 suggest that downregulation of p53 protein partially
152 rescues DOX-enhanced MCF-7 cell killing by CPA.

150 Discussion
151 In the present study, the effects of antimicrobial agent
152 doxycycline (DOX) on cyclophosphamide (CPA)-mediated
153 MCF-7 breast carcinoma cell death, both in vivo and in
154 vitro, were investigated. The results indicate that DOX
155 potentiates in vivo tumor regression activity and also
156 enhances in vitro cell killing of MCF-7 by CPA. In all the
157 aspects studied, DOX enhanced CPA cytotoxic activity both
158 in vivo and in vitro appears identically regulated, except for
159 comparatively lowered Bcl-2 levels in nude mice model
160 which could be due to additional effect of bioactivation of
161 CPA by liver-specific enzymes.
162 This is the first report demonstrating the potentiation of
163 antitumor activity of CPA on solid tumor cells by a non-
164 anticancer drug and one of the few on drugs combined with
165 high-dose regimen CPA to increase its therapeutic index.
166 DOX, a water-soluble analogue of tetracycline, is used for
167 treating a variety of infectious diseases in cancer patients
168 (Liston and Koehler, 1996; Moreno et al., 1994). It is not
169 only a well-tolerated antibiotic but also has been exploited
170 as inhibitor of proliferation and inducer of apoptosis, causes
171 cell cycle arrest in cultured and as an antitumor agent in
172 several tumor systems (van den et al., 1986, 1988). DOX
173 has been shown to inhibit the activity of 72- and 92-kDa
174 type IV collagenase (MMPs) secreted by bone-metastasizing
175 cells by 79–87%. Using tumor cell lines relevant to bone
176 metastases, that is, PC-3, MDA-MB-231, Hs696, B16/F1, it
177 has been demonstrated that tetracycline and derivatives of
178 tetracycline, namely, DOX and minocycline, also induced
179 cytotoxicity (Duivenvoorden et al., 2002; Fife et al., 1998).
180 Moreover, DOX treatment in an experimental bone meta-
stasis mouse model of human breast cancer MDA-MB-231 cells resulted in a 70% reduction in total tumor burden compared with placebo-controlled animals (Duivenvoorden et al., 2002). It is also being investigated as an antiangiogenic agent for treatment of cancer (Gilbertson et al.)

MCF-7 cell line to CPA has been investigated of liver-derived activated drug metabolites (Chen et al., 1996; Schwartz and Waxman, 2001). Also, with proved carcinogenic and teratogenic side effects (Cohen et al., 1992), it has become applicable to target combination therapy to lower the dose of such drugs. Potentiation of antitumor and cytotoxic effect of CPA by addition of thalidomide. DOX, and bis-indole alkaloid has been reported. Thalidomide and DOX have been shown to potentiate antitumor effect of CPA in murine tumor models using Cohen 38 tumor cells and L 1210 leukemia cells (Ding et al., 2002; Pakulska, 1992). In addition, Yuebeihuanke, a bis-indole alkaloid, is reported to potentiate the cytotoxic effect of CPA on MCF-7 cells (Leung et al., 2000). Our observation that DOX can potentiate CPA-induced regression of solid tumor cells in mice further supports the usefulness of DOX as a potential candidate to be used in combination chemotherapeutic treatment for cancer.

Our results indicate enhanced upregulation of p53 and p53-regulated proapoptotic protein Bax upon treatment of MCF-7 cells with a combination of DOX and CPA in vitro and in vivo. Uregulation of p53 and Bax correlated with tumor regression. Additionally, downregulation of antiapoptotic Bcl-2 was observed in animals treated with CPA and CPA plus DOX compared to untreated and DOX-treated animals. DOX enhanced cytotoxic activity of CPA in vitro results in increase in cellular population undergoing apoptosis. Also, DOX in a dose-dependent manner increases CPA-mediated MCF-7 cell death. Activation of p53 promoter and increase in p53 protein following DOX plus CPA treatment together suggest p53 involvement. Moreover expression of antisense p53 in MCF-7 cells also prevents DOX enhanced CPA-mediated cell death indicating involvement of p53. Furthermore, increase in mRNA as well as protein levels of pro-apoptotic protein Bax in these cells correlates with increased Bax mRNA detected in tumors of animals treated with DOX and CPA together. Therefore, enhancement of cytotoxic potential of CPA by DOX is due to increased p53 and Bax protein levels resulting in tumor regression by CPA in presence of DOX in vivo. This study also highlights relevance of p21 (WAF1/CIP1) independent apoptosis and significance of alterations in Bax/Bcl2 ratio favoring apoptosis and antitumor effect both in vitro as well as in vivo (Huang et al., 2003). Our results are consistent with those reported for combination of chemotherapeutic agents with CPA against breast cancer cells (Freyre et al., 2000). These observations suggest that combination chemotherapy may derive its efficacy partly through coordinated regulation of specific gene products associated with apoptosis. Moreover, characterization of molecular events that underlie susceptibility of specific tumor cells to combination chemotherapeutic regimens may lead to additional improvements in treatment strategies for cancers.
A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis.


Bystander Killing of Breast Cancer MCF-7 Cells by MDA-MB-231 Cells Exposed to 5-Fluorouracil Is Mediated via Fas

Rishi Raj Chhipa and Manoj Kumar Bhat*
National Centre for Cell Science, Ganeshkhind, Pune 411 007, India

Abstract • The major drawback with cancer therapy is the development of resistant cells within tumors due to their heterogeneous nature and due to inadequate drug delivery during chemotherapy. Therefore, the propagation of injury ("bystander effect" (BE)) from directly damaged cells to other cells may have great implications in cancer chemotherapy. The general advantage of the bystander cell killing phenomenon is the large therapeutic index that can be achieved. Experiments suggest that this phenomenon is detected in radiation therapy as well as in gene therapy in conjunction with chemotherapy. In the present study, we developed an original in vitro model dedicated to the exploitation of bystander cytoltoxicity-induced during breast carcinoma chemotherapy. In brief, we investigated this perpetuation of injury on untreated bystander, MCF-7 breast cancer cells which were coplated with 5-fluorouracil (5-FU)-treated MDA-MB-231 breast cancer cells. To achieve this goal, a specific in vitro coculture model which involved mixing of aggressive MDA-MB-231 breast cancer cells with enhanced green fluorescent protein (EGFP) expressing stable clone of non-metastatic MCF-7 breast cancer cells (MCF-EGFP), was used. A bystander killing effect was observed in MCF-EGFP cells cocultured with MDA-MB-231 cells pretreated with 5-FU. The striking decrease in MCF-EGFP cells, as detected by assaying for total GFP intensity, is mediated by activation of Fas/FasL system. The implication of Fas in MCF-EGFP cell death was confirmed by using antagonistic anti-FasL antibody that reverses bystander cell death by blocking FasL on MDA-MB-231 cells. In addition, inhibition of CD95/Fas receptor on the cell surface of MCF-EGFP cells by treatment with Pifithrin-alpha, a p53 specific transactivation inhibitor, partially abrogated the sensitivity of bystander MCF-EGFP cells. Our data, therefore, demonstrates that the Fas/FasL system could be considered as a new determinant for chemotherapy-induced bystander cell death in breast cancers.

Key words: bystander effect; p53; Fas; 5-FU; breast cancer

Solid tumors are difficult to treat, though surgery is performed where it is possible, chemotherapy is mostly the choice of treatment. One of the major limitations of chemotherapy is the inability of anticancer agents to induce cytotoxicity due to inaccessibility to certain cells in a tumor. Moreover, histopathological heterogeneity in human tumors is a well-documented phenomenon. It is now widely admitted that breast carcinoma is a genetically and clinically heterogeneous disease as it contains different clones of tumor cells arising from continual differentiation of transformed progenitor cells [Symmans et al., 1995; Konemann et al., 2000; Sharifi-Salamatian et al., 2004; Li and Rosen, 2005]. Since cancer now is considered more as a deficiency of apoptosis rather than a mere proliferation issue [Guchelaar et al., 1997], additional mechanisms which induce or enhance cell death during cancer chemotherapy will be beneficial for the better outcome of the treatment. Though determining the factors involved in drug response is a new challenge in modern cancer chemotherapy [Van Triest et al., 2000], a new phenomenon called "The Bystander Effect" (BE) has been attributed in several investigations for the propagation of injury in cancer therapy. The name was initially borrowed from the gene therapy field, where it...
usually referred to the killing of several types of tumor cells by targeting only one type of cell within a mixed population [Cheng et al., 1999].

The BE is a chemo and radiobiological phenomenon that has come to the force recently. It describes the ability of cells affected by an agent to convey manifestations of damage to other cells neither directly targeted by the agent nor necessarily susceptible to it per se. Thus, the BE is elicited indirectly by communication between cells which are directly affected by agent and those that are not. While the term BE may cover a variety of distinct mechanisms, the common denominator is cellular interaction, either to increase or decrease viability of non-target cells [Djordjevic, 2000]. The data available concerning the BE falls into two quite separate categories. First, there are experiments involving the transfer of medium from treated cells (effector cells), to untreated cells (target cells) resulting in biological effect on later cells. Second, there is the use of specifically treated effector cell population and BEs are studied in the target cells cocultured subsequently with these effector cells. Several reports have described utilization of mixed population of cells and cocultures to study the BEs [Kagawa et al., 2001; Shao et al., 2005]. These cocultures were constituted by mixing either homogenous or heterogeneous population of cells (Hofmunn and Blau, 1997; Tanaka et al., 2001). BE has been shown for cell lethality, chromosomal aberrations, and cell cycle delay (Scott, 2004). The type of cells involved and type of treatment appear to be an important determinant. Therefore, the bystander studies suggest that biological effects of any anticancer therapy are larger than the assumed outcome of these treatments.

The bystander cytotoxicity using gene therapy in conjunction with chemotherapy for example, Herpes Simplex Virus Thymidine kinase gene utilized for activation of prodrug Ganciclovir has been described in several investigative reports [Grignet-Debrus et al., 2000; Mesnil and Yamasaki, 2000]. Additionally, the role of factors like p53, TRAIL gene, and Cytochrome P-450 in bystander cell death in cancer cells has been reported by some laboratories [Xu et al., 1997; Zhou et al., 2000; Kagawa et al., 2001]. Moreover, this effect has also been demonstrated in cancer cells exposed to both high- and low-energy radiations in several separate experimental models [Albanese and Dainiak, 2000; Hall, 2003; Mothersill and Seymour, 2004]. It has been demonstrated that following a low dose of alpha particles, a larger population of cells manifested biological damage than those actually have been hit by alpha particles [Nagasawa and Little, 1992; Hall, 2003]. Chemotherapeutic drugs can induce apoptosis and upregulate death ligands or their receptors which may subsequently play a significant role in death signal amplification via BE in a mixed population of cells. Significant number of investigations have shown apoptosis mediated via Fas/FasL system in cancer cells treated with anticancer agents [Priesen et al., 1999; Mo and Beck, 1999]. However, the implication of chemotherapeutic stress-induced bystander cell death has been least investigated regimen in cancer biology.

In the present study, we developed clones from breast cancer cells, MCF-7 and MDA-MB-231, (MCF-EGFP and MDA-MB-231-EGFP respectively), which stably expressed enhanced green fluorescent protein. We utilized them as an expeditious tool to study bystander cell death phenomenon in a mixed culture assays. We investigated chemotherapeutic agent 5-fluorouracil (5-FU) or Carboplatin (Carb) induced cytotoxicity either in MDA-MB-231 or MCF-7 cells (drug treated cells were termed as “effector cells”) on bystander MCF-EGFP or MDA-MB-231-EGFP cells (EGFP expressing cells which were not treated with drugs were termed as “target cells”). The BE-mediated toxicity on target cells was evaluated by measuring the green fluorescence intensities. We, for the first time, demonstrated that MCF-EGFP target cells die when cocultured with MDA-MB-231 effector cells that were pretreated with 5-FU. This phenomenon is both drug and cell type specific and is dependent on membrane bound death receptor/death ligand, Fas/FasL system. To our knowledge, no comparable systematic study on the efficacy of chemotherapy-induced bystander killing targeted towards cancer treatment exists.

MATERIALS AND METHODS

Reagents and Antibodies

Sources of materials were as follows: 5-fluorouracil (5-FU), Carboplatin (Carb), 8-Bromocyclic-AMP (BCMP), and Pifithrin alpha (PFTa) were purchased from Sigma, MO. Carb and 5-FU were dissolved in sterile water to prepare 50 mM stock. PFTa was dissolved in
Fas Induced Bystander Killing in Breast Cancer Chemotherapy

DMO, BCM P was dissolved in sterile water to prepare 1 M stock solution. Antagonistic anti-FasL antibody and anti TRAIL antibodies (BD Biosciences, CA) were reconstituted in sterile PBS as 1 mg/ml stocks.

Cell Cultures and Development of MCF-EGFP and MDA-MB-231-EGFP Cell Lines

Human breast cancer lines MCF-7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were obtained from ATCC (Manassas, VA) and maintained in our in-house National Cell repository. Cells were routinely cultured in DMEM and F12K (1:1) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, UH), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, CA), at 37°C with 5% CO₂. The MCF-7 and MDA-MB-231 cells were separately transfected with pEGFPN1 plasmid (Clontech, CA) by calcium phosphate method and selected on G418 (USB, OH) 800 and 600 μg/ml, respectively. Subsequently, the cells were maintained in medium containing G418 (100 μg/ml).

Bystander Cytotoxicity

We designed an in vitro coculture experiment to evaluate whether the cytotoxicity induced by commonly used chemotherapeutic agents such as 5-FU or Carb, either in MDA-MB-231 or MCF-7 breast cancer cells (drug treated cells were termed as effector cells) leads to death of cocultured bystander MCF-EGFP or MDA-MB-231-EGFP cells (EGFP expressing breast cancer cells which were not treated with drugs were termed as target cells). The bystander EGFP positive target cells were evaluated for cell death by quantification of total live fluorescence by microfluorimetry with an absorbance at 488 nm and an emission at 510 nm (Fluoroskan Ascent FL, Labsystems, Finland). The fluorescence intensities of bystander target cells cocultured with untreated effector cells were plotted as 100%. Briefly, the exponentially growing effector cells (50,000 per well) were plated in a 24-well tissue culture plate and after allowing them to adhere for 24 h, cells were treated with 100 or 500 μM of 5-FU or Carb for additional 24 h. Subsequently, effector cells were washed twice with medium and the target cells were cocultured (50,000 per well) without or with PFTa (20 μM), DMSO, FasL or TRAIL antagonistic antibodies (500 ng/ml each), or BCPM (1 mM) treatments, as required for the experiments. After 48 h of growth in coculture, both target and effector cells were trypsinized and quantituated for EGFP intensity of surviving fraction of target cells, as described above. In the investigations where conditioned media from effector cells was assayed for its toxicity on bystander target cells, the effector cells were pretreated with the anticancer agent for 24 h. After washing twice, the effector cells were incubated further in complete medium for 48 h and subsequently, this medium was used for culturing target EGFP positive cells for 48 h before reading fluorescence.

Subcellular Fractionation

The cytosolic and membrane fractions were obtained by ultracentrifugation [Nakajima et al., 2000]. Briefly, cells were removed from plates using rubber policeman and homogenized in ice cold buffer containing 20 mM Tris-HCl, pH 7.6, 10 mM EDTA, 10 mM EGTA, 1 mM NaHCO₃, 5 mM MgCl₂, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na₃VO₄, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1.5 mM pepstatin. Then homogenate was centrifuged at 100,000 g for 60 min at 4°C and supernatant was collected as cytosolic fraction. The pellet was washed with the same buffer, resuspended in buffer containing 1% Triton X-100, and recentrifuged at 100,000g for 60 min at 4°C. The resultant supernatant was collected as the membrane fraction. The fractions thus obtained were immunoblotted as described below.

Western Blot Analysis

Following treatments, cells were washed thrice with ice cold PBS and lysed in 100 μl of ice-cold lysis buffer (20 mM HEPES pH 7.4 containing 1% NP-40, 2 mM EGTA, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM TPCCK, 2 mM Na₃VO₄, and protease inhibitor cocktail tablet) per 1 × 10⁶ cells. Samples were boiled in SDS sample buffer for 10 min followed by separation on an SDS–PAGE. Equal amount of protein samples (75 μg) were resolved on 10–12% SDS–polyacrylamide gel and then transferred onto nitrocellulose membranes (Amer sham, Aylesbury, UK). The membranes were probed with antibodies against p53, estrogen receptor-
alpha, Fas receptor, caspase-8, and β-actin (Santa Cruz Biotechnology, CA). The immunoblots were detected by enhanced chemiluminescence (ECL) reagent (New England Biolabs, MA).

Reverse Transcription Polymerase Chain Reaction Analysis for FasL Expression in MDA-MB-231

Total cellular RNA from treated and untreated cells was extracted using TRizol™ reagent (Invitrogen Life Technologies, CA), according to the manufacturer's instructions. Five micrograms of total RNA and oligo (dT)$_{12-18}$ primer was taken in diethylpyrocarbonate (DEPC)-treated water. cDNA synthesis was initiated using 200 U of M-MLV reverse transcriptase (Invitrogen Life Technologies), under conditions recommended by the manufacturer and the reaction was allowed to proceed at 37°C for 50 min. Reaction was terminated by heating at 70°C for 15 min. Each RT-PCR contained 10% of cDNA, 20 mM of each primer in 20 mM Tris-HCl (pH 8.4) containing 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTP mix, and 1 U of platinum Taq DNA polymerase (Invitrogen Life Technologies) in a final volume of 20 µl. After an initial denaturation for 2 min at 95°C, 30 cycles of denaturation (94°C for 1 min), annealing (for 1 min), and extension (72°C for 1 min) were performed on a DNA thermal cycler (Techne, Cambridge, UK) with a final extension for 10 min at 72°C. The primer pairs used were as follows: FasL 5'-GTC AAT CTT GTA ACA ACC TGG-3' (F) 5'-ACA ACA TTC CTG GTG CCT G-3' (R) and β-actin 5'-ATC TGG GAC CAC ACC TTC TAC AAT GAG CGT CCC CAT ACT TCC GCC TCC ATC CAT ATC TGC-3' (F) 5'-CTG CAT CTC ACC TCC TTT TTC TCG GTG ACT CCC TGC TGC ATC CAC ATC TGC-3' (R). The annealing temperatures for FasL and β-actin PCR were 57 and 55°C, respectively.

Apoptotic Death Detection by Annexin V-PE Staining

To evaluate whether the death of target cells (MCF-EGFP) is due to induction of apoptosis, we carried out annexin V-PE staining and cells were analyzed by flow cytometry. MCF-EGFP cells were cultured alone, cocultured with untreated effector MDA-MB-231 cells or cocultured with drug-treated effector MDA-MB-231 cells according to the methodology earlier. Briefly, cells were washed with PBS and stained with annexin V-PE according to the manufacturer's protocol (ApoAlert Annexin V-PE Apoptosis Kit; Clontech, CA). Washed cells were subjected to FACSscan flow cytometer (Becton Dickinson Gmbh, Heidelberg, Germany). Forward scatter (FSC) and orthogonal scatter (SSC) were collected using linear amplification. Annexin V-PE and EGFP fluorescence was collected using log amplification and 1,000 events were recorded. Cells were analyzed using quadrant statistics in an annexin V-PE versus EGFP dual parameter histogram. Cell populations were expressed as percentages.

Statistical Analysis

Data are expressed as the mean of three independent results. Statistical comparisons are made using Student's t-test and P-value <0.05 was considered as significant.

RESULTS

Chemotherapy Induced Bystander Cytotoxicity in the Mixed Cultures of Breast Carcinoma Cells

Bystander cytotoxicity has been projected essentially as a radiobiological as well as gene therapy associated phenomenon in cancer biology, thus, our quest was to investigate whether it also occurs during cancer chemotherapy. We developed stable cell lines which constitutively express EGFP. The MCF-EGFP and MDA-MB-231-EGFP cells lines did neither exhibit any significant alterations in their growth properties nor do they exhibit any alternations in sensitivity to chemotherapeutic drugs as compared to parental cells (data not shown). We established an appropriate model by treating MDA-MB-231 or MCF-7 breast cancer cells with 5-FU or Carb and subsequently coculturing either MCF-EGFP or MDA-MB-231-EGFP cells. Results thus obtained demonstrated that 5-FU-treated MDA-MB-231 induced bystander killing in MCF-7 cells as detected by measuring total GFP intensity (Fig. 1B) or by observing cells under a fluorescent microscope (Fig. 2, panel e). By quantification of green fluorescence; it was also established that MCF-7 cells treated with 5-FU or Carb at any of the two doses (100 and 500 µM), did not induce bystander killing in either MCF-EGFP (Fig. 1A) or in MDA-MB-231-EGFP cells (Fig. 1C). This observation was supported by fluorescent photomicrographs of target bystander MCF-EGFP (Fig. 2, panels b and c) or in target bystander MDA-MB-231-EGFP cells (Fig. 2, panels h and l). The fluorescent photomicrographs are representative of observations where 500 µM of drugs...
were used. Similarly, 5-FU and Carb-treated MDA-MB-231 did not induce bystander killing in MDA-MB-231-EGFP cells (Fig. 1D). The representative photographs (Fig. 2, panels k and l) from the coculture were in support of fluorescence intensity data. However, MDA-MB-231 cells treated with varying doses of 5-FU induced bystander killing of MCF-EGFP cells in a dose dependent manner (*P < 0.05 vs. untreated effector cells). 5-FU at a dose of 100 and 500 μM led to decrease in GFP intensity by 15 and 40%, respectively (Fig. 1B). In contrast, no such bystander killing of MCF-EGFP cells was detected with Carb treated MDA-MB-231 cells (Figs. 1B and 2, panel f). Data presented here clearly demonstrates that 5-FU-treated effector MDA-MB-231 cells induce bystander killing of target MCF-EGFP cells.

The Bystander Cytotoxicity in Target MCF-7 Cells Is Mediated Via Membrane Bound Fas Ligand Expressed on the Effector Cells

Since 5-FU-treated MDA-MB-231 induced bystander killing in MCF-7 cells, we next explored the molecular alterations that may be involved in the cell killing. We investigated the involvement of death ligands like, Fas ligand (FasL) and TRAIL. In several studies, both these ligands have been reported to be involved in bystander cell death [Kagawa et al., 2001; Ciccolini et al., 2002; Hyer et al., 2003]. To investigate the involvement of death ligands in bystander killing of MCF-7 cells by 5-FU (500 μM)-treated MDA-MB-231 cells, we performed coculture experiments in the presence of FasL and TRAIL antagonistic antibodies as described in the materials and methods. In brief, the effector cells were 5-FU treated, washed, and the target cells were plated in media in the presence of antagonistic antibodies for 48 h. As shown in Figure 3A, when the bystander EGFP positive cells were coplated with the treated effector cells in presence of antagonistic FasL antibody, the EGFP intensity of target cells was similar to that of cells plated with control cells, suggesting involvement of FasL in the bystander cell killing (*P < 0.05 vs. drug-treated effector cells). Under similar experimental

![Figure 1](image-url)

**Figure 1.** Coculture experiments to evaluate chemotherapy-induced bystander killing in breast cancer cells. A-C. Bystander killing effect mediated by effector MCF-7 cells treated with 5-FU and Carb 100 and 500 μM doses for each drug for 24 h, towards the target MCF-EGFP and MDA-MB-231-EGFP cells, respectively. B, D. Bystander killing effect mediated by effector MDA-MB-231 cells treated with 5-FU and Carb 100 and 500 μM doses for each drug for 24 h, towards the target MCF-EGFP and MDA-MB-231-EGFP cells, respectively. Data represent mean ± SD from three independent experiments (*P < 0.05 vs. untreated effector cells).
conditions when TRAIL antagonistic antibody was utilized, no consequent increase in the EGFP intensity of target cells was observed, suggesting its non-involvement. The bystander transmission of cell death signal may be either by direct interaction of membrane bound Fas ligand of effector cells and Fas receptor of target cells following cell-to-cell contact or by some soluble FasL (Schneider et al., 1998) released by stressed effector cells in the cocultures. Therefore, complementary studies were carried out which involved the transfer of medium from the treated effector cells to the target cells. As shown in Figure 3B, when the target cells were grown in the media obtained from the treated effector cells, no decrease in EGFP intensity was observed, indicative of complete absence of cytotoxic soluble factor in the culture supernatants ($P \leq 0.05$ vs. drug-treated effector cells). Moreover, separate experiments showed that GJIC (gap junction-mediated intercellular communication) was also not involved in the transmission of cytotoxicity from effector to target cells as the specific GJIC enhancer, BCPM, did not enhance the killing (Fig. 3B) of MCF-EGFP cells in coculture [Azzam et al., 2001, 2003; Chipman et al., 2003]. Taken together, these results imply that membrane bound FasL-induced death signal is indeed involved in decrease in the EGFP intensity of bystander target cells.

Membrane Bound FasL Expression Is Upregulated in 5-FU-Treated MDA-MB-231 Effector Cells

We next investigated whether FasL and its cognate Fas receptor expression is upregulated in effector MDA-MB-231 cells following drugs treatment. MDA-MB-231 cells were treated with 500 nM each of 5-FU and Carb separately, and membrane fractions were prepared and analyzed for FasL and Fas receptor expression. Interestingly, as shown in Figure 3C, in MDA-
MB-231 cells, treatment with 5-FU for 24 h leads to significant increase in FasL levels which was enhanced further if the cells are allowed to grow for additional 24 h in drug-free media following the treatment. Under similar experimental conditions, no increase in FasL was observed in Carb-treated MDA-MB-231 cells. Moreover, no alterations in Fas receptor were detected in MDA-MB-231 cells treated with either drug. Further, the increase in FasL at the level of transcription was confirmed by RT-PCR analysis of RNA from 5-FU (500 μM)-treated MDA-MB-231 cells (Fig. 3D). Overall, these results indicate that in MDA-MB-231 cells, 5-FU treatment not only enhanced FasL mRNA, but it also resulted in increase in its localization on the membrane thereby facilitating bystander killing of target MCF-EGFP cells.

p53 and Fas Receptor Contributes to the Bystander Killing of Target MCF-EGFP Cells

The biological effects of FasL are mediated by the expression of Fas receptor (FasR) protein expression in MDA-MB-231 cells (lane 1) untreated, (lane 2) treated with 5-FU or Carb for 24 h, and (lane 3) treated with 5-FU or Carb for 24 h and further allowed to grow for 24 h in the absence of drug. Relative levels were measured by densitometry with control normalized to 100. D: PCR analysis of FasL mRNA expression by MDA-MB-231 cells and 5-FU treated MDA-MB-231 cells. The positions of β-actin and FasL transcripts are shown.

Fig. 3. Bystander cell killing is due to enhanced FasL expression on effector cells. A: Effect of antagonistic antibodies to FasL and TRAIL on the cell death of bystander MCF-EGFP cells (*P< 0.05 vs. drug-treated effector cells). B: Effect of conditioned media and G418 enhancer. Bomp GFP intensity was taken as 100% for cocultures where effector cells were not treated with 5-FU. Error bars represent the standard deviation of the mean from the results of experiments done in triplicate (*P < 0.05 vs. drug-treated effector cells). C: Western blot analysis for FasL and Fas receptor

MB-231 cells, treatment with 5-FU for 24 h leads to significant increase in FasL levels which was enhanced further if the cells are allowed to grow for additional 24 h in drug-free media following the treatment. Under similar experimental conditions, no increase in FasL was observed in Carb-treated MDA-MB-231 cells. Moreover, no alterations in Fas receptor were detected in MDA-MB-231 cells treated with either drug. Further, the increase in FasL at the level of transcription was confirmed by RT-PCR analysis of RNA from 5-FU (500 μM)-treated MDA-MB-231 cells (Fig. 3D). Overall, these results indicate that in MDA-MB-231 cells, 5-FU treatment not only enhanced FasL mRNA, but it also resulted in increase in its localization on the membrane thereby facilitating bystander killing of target MCF-EGFP cells.

p53 and Fas Receptor Contributes to the Bystander Killing of Target MCF-EGFP Cells

The biological effects of FasL are mediated by the expression of Fas receptor (Fas) on the cell surface and p53 plays a major role in directly regulating its expression (Bennett et al., 1998; Muller et al., 1998). Therefore, the bystander cell killing of target cells MCF-EGFP was examined for its dependency on tumor suppressor p53. The target cells were cocultured on 5-FU-treated effector cells in the absence or presence of PFTα, a potent and specific inhibitor of p53 transactivity. Interestingly, as shown in Figure 4A, in the presence of PFTα, the EGFP intensity of target cells was significantly more than either DMSO treated or experimental cells, indicating the survival of target cells (*P < 0.05 vs. drug-treated effector cells). Subsequently, we next examined whether inhibition of p53 activity alters the levels of Fas receptor on the membrane of the target cells. As shown in Figure 4B, treatment of cells with PFTα leads to decrease in the expression levels of Fas receptor when compared to the control cells or DMSO-treated cells. As compared to control or DMSO-treated cells, PFTα treatment causes almost 45% decrease in Fas receptor
level in target cells (Fig. 4B, lanes 1 and 2 vs. lane 3). Under the similar treatment conditions, no significant decrease in either the p53 or membrane associated estrogen receptor alpha (ERα) was detected. These results indicate that presence of Fas receptor and p53 activity is involved in mediating cell death in the target bystander cells.

Fas/FasL Activates Procaspase-8 in Target Cells

Caspases play a major role in execution of death signal induced by various apoptotic stimuli. Caspase-8 is a part of this growing family of cysteine proteases that were shown to be involved in many forms of apoptosis. Caspase-8 functions downstream of the death receptor-mediated pathways involving CD95/Fas and tumor necrosis factor alpha receptor [Ashkenazi and Dixit, 1998]. Moreover, all caspases are synthesized as inactive proenzymes that have to be activated by proteolytic cleavage. We determined the extent of conversion of procaspase-8 to active caspase-8 by immunoblot analysis. As shown in Figure 5A, significant decrease in the cellular procaspase-8 levels was observed in the lysate prepared from cocultures of 5-FU-treated effector cells and target cells when compared to the protein levels in cocultures where no 5-FU was added to effector cells (lane 5 vs. lane 3). In the coculture conditions, the effector and target cells were plated in a 1:1 ratio, as described in Materials and Methods, and equal protein was resolved in each lane. The decrease in procaspase-8 has been attributed to its activation and corresponds to conversion of pro-enzyme to its active subunits following association of activated Fas and death-inducing signaling complex [Scaffidi et al., 1998]. The unchanged procaspase-8 level in lane 4 indicates that the decrease in procaspase-8 levels is not contributed by drug-treated effector cells. Moreover, no decrease in procaspase-8 levels was detected in untreated MCF-7 and MDA-MB-231 cells (lane 1 and lane 2, respectively). As a positive control, MCF-7 cells were treated with TNF-α (lane 6). TNF-α is an activator of caspase-8-dependent apoptotic pathway [Benoit et al., 2004], and it leads to decrease in procaspase-8 levels (lane 6). These results imply that activation of Fas/FasL pathway is responsible for decrease in procaspase-8 levels in the target cells.

Apoptotic Cell Death in Bystander Target Cells

Because our results demonstrated the involvement of FasL/Fas as well as decrease in procaspase-8, we investigated if this would lead to the induction of apoptosis in bystander target cells. To answer this question, we took advantage of measuring cells simultaneously for both phycoerythrin (PE)-conjugated annexin V staining and EGFP positive counts by FACS analysis. The percentage of effector and target cells undergoing apoptosis was determined by their PE-conjugated annexin V staining. As shown in Figure 5B, panel d, when target MCF-

Fig. 4. p53 and Fas receptor are involved in target cell death.
A: Effect of PFTs, which specifically inhibits wild-type p53 activity, on MCF-ECFP cell survival. Error bars represent the mean ± SD from the results of triplicate experiments (p < 0.05 vs. drug-treated effector cells). The GFP intensity was taken as 100% for cocultures where effector cells were not treated with 5-FU. B: A representative western blot showing levels of Fas receptor (FasR) expression on the cell surface of target MCF-ECFP cells when treated with PFTs. Relative levels were measured by densitometry with control normalized to 100 and compared with characteristic estrogen receptor alpha (ERα) levels on the cell membrane.
Fas Induced Bystander Killing in Breast Cancer Chemotherapy

**Fig. 5.** Caspase-8 activation leads to apoptotic cell death in target cells. A: Western blot analysis for total procaspase-8 levels. In untreated MCF-7 cell (lane 1); untreated MDA-MB-231 cells (lane 2); cocultured MCF-7 with untreated MDA-MB-231 cells (lane 3); 5-FU-treated MDA-MB-231 cells (lane 4); cocultured MCF-7 cells with 5-FU-treated MDA-MB-231 cells (lane 5); and MCF-7 cells alone treated with TNF-α (10 ng/ml), which served as positive control for procaspase-8 cleavage (lane 6). B: Apoptotic cell death in bystander target MCF-EGFP cells. Histograms for effector and target cell populations alone or in coculture are shown after annexin V-PE staining. Untreated MDA-MB-231 cells (panel a), untreated MCF-EGFP cells (panel b), 5-FU-treated MDA-MB-231 cells (panel c), MCF-EGFP cells cocultured with untreated MDA-MB-231 cells (panel d), and MCF-EGFP cells cocultured with 5-FU-treated MDA-MB-231 cells (panel e) are shown with annexin-V-PE positive counts indicated as percentages of apoptotic cells. The right quadrants (panel e) represent proportion of apoptotic bystander MCF-EGFP cells.

EGFP cells were cocultured with untreated effector MDA-MB-231 cells, barely about 2% of target MCF-EGFP cells were positively stained for annexin V-PE. However, when the target MCF-EGFP cells were cocultured with 5-FU treated effector MDA-MB-231 cells, about 10% of these GFP positive cells were positive for annexin V-PE (Fig. 5B, panel e). These results clearly indicate induction of apoptosis in bystander target cells. The untreated effector as well as target cell by themselves had a very small basal population of apoptotic cells (Fig. 5B, panels a and b, respectively). In case of effector cells alone exposed to 5-FU, more than 25% cells were apoptotic as analyzed by annexin V-PE positive counts (Fig. 5B, panel c). This was identical to the values obtained when 5-FU treated effector cells were cocultured with the target cells (Fig. 5B, panel c). The apoptosis of effector cells was not affected when they were cocultured with target cells.

**DISCUSSION**

Killing of tumor cells by cytotoxic therapies, for example, chemotherapy, radiotherapy, immunotherapy, or suicide gene therapy is predominantly mediated by triggering injury in cancer cells. Cancer chemotherapy suffers major drawback as chemoresistant cells develop within the tumors due to their heterogeneous nature, most importantly in response to therapy under different treatment regimens, and due to inadequate drug delivery methods. Therefore, it is becoming apparent that better understanding of mechanism of drug-induced injury to cancer cells will have profound effect on cancer treatment and management.

Breast cancers are heterogeneous in nature and often progress from a low-invasive estrogen sensitive phenotype to high-invasive, estrogen insensitive phenotype. MCF-7 cell line is prototype for estrogen sensitive breast cancer cells,
representative of relative early stages in breast cancer development. The data presented here demonstrates that 5-FU-treated aggressive breast cancer cells MDA-MB-231, induce bystander killing of MCF-7 breast cancer cells, even though they are not exposed directly to the drug itself. No such bystander cell killing was observed with Carb, another commonly used drug for treating solid tumors like breast cancer. Therefore, 5-FU may potentially be of more therapeutic value than other chemotherapeutic drugs used in the management of aggressive breast cancers because of its ability to initiate bystander cell killing.

It has been demonstrated in earlier studies that following alpha particles irradiation, a larger population of cells were affected than those actually have been hit directly by alpha particles [Nagasawa and Little, 1992; Hall, 2003]. Additionally, there are reports on the enhancement of the Herpes simplex virus thymidine kinase/ganciclovir induced BE and its antitumor efficacy in vivo by pharmacologic manipulation of gap junctions. Furthermore, enhanced cell killing by gene therapy used for prodrug activation also underscored the enhanced therapeutic efficacy of BEs [Touraine et al., 1998; Zhou et al., 2000]. Moreover, since chemotherapeutic drugs can induce apoptosis and upregulate death ligands (TNF, FasL, and TRAIL) or their receptors that may subsequently play a significant role in death signal amplification via BE in a mixed population of cells [Petak and Houghton, 2001]. Finally, the death receptor based amplification of the therapeutic response may be clinically meaningful since it may critically affect the time required for execution of the death program and death receptor based immune clearance of tumor cells [Micheau et al., 1997; Solary et al., 1998; Poulaki et al., 2001]. Taken together, these studies indicate that the bystander phenomenon has great implications in cancer therapies due to the large therapeutic index that can be achieved.

Determining the factors involved in drug response is a new challenge in modern chemotherapy. Therefore, to investigate the molecular pathways that may be contributing to bystander cell death, we explored the involvement of FasL, Fas and role of tumor suppressor p53. Even though number of investigations have shown induction of apoptosis in a variety of cancer cell types treated with anticancer agents like doxorubicin, cytarabine, etoposide (VP-16), cisplatin, bleomycin, as well as anti-metabolites such as methotrexate and 5-FU [Houghton et al., 1997; Micheau et al., 1997; Friesen et al., 1999; Mo and Beck, 1999; Tillman et al., 1999] is mediated via Fas/FasL system, none have shown its involvement in bystander killing of heterogeneous breast cancer cells. Moreover anticancer drugs are known to enhance FasL expression [Micheau et al., 1997]. FasL, which functions as an autocrine/paracrine mediator of apoptosis induced by DNA damaging anticancer chemotherapeutic agents, is a member of the TNF superfamily. It induces apoptosis in susceptible cells upon cross-linking to its own receptor, FAS (Apo-1/CD95).

We observed that upregulation of FasL in MDA-MB-231 exposed to 5-FU induces bystander killing of MCF-7 cells, whereas vice-versa no such effect is observed either in any mixed culture assay or when only single cell type is utilized for the experiment. These results are in agreement with the reports that higher expression of FasL in vivo has been found in tumors with more aggressive clinical behavior as that of MDA-MB-231 cells or tumors with poor histological prognosis, supporting a role for this cytolytic molecule in cancer progression [Gratas et al., 1998; O'Connell et al., 1999]. It has also been reported that MDA-MB-231 cells are resistant to Fas-induced apoptosis, whereas MCF-7 cells are Fas-sensitive [Toillon et al., 2002]. The conditional media obtained from the treated cells as well as the application of gap junction enhancer did not enhance MCF-EGFP cell death. The results suggest the involvement of membrane associated Fas/FasL rather than soluble FasL.

In addition to FasL/Fas pathways, it has been well documented that p53 and factors dependent on p53 potentiate cell death in chemo and radiation therapy of various cancer models [Komarova et al., 1998; Nishizaki et al., 1999]. Especially in the context of p53-mediated gene therapy, adenovirally delivered p53 (AdWTp53) has been evaluated for induction of in vitro as well as in vivo bystander cytotoxicity [Rizk et al., 1999]. Additionally, p53, in addition to its intrinsic antiproliferation activity, can be both, the cause and the target of BE by inducing export of growth suppressive stimuli from damaged cells to neighboring cells. Consistent with this observation, a p53-dependent accu-
mulation of factors, which causes growth inhibitory effects in a variety of cell lines, were found after gamma irradiation in the media from established and primary cell cultures and in the urine of irradiated mice [Komarova et al., 1998]. Thus in our experiments, the chemotherapy-induced BE was also examined for its dependency on wild-type tumor suppressor p53 protein expression in the target MCF-EGFP cells [Ozbun and Butel, 1995]. We demonstrate that cell surface expression of Fas receptor was downregulated by inhibition of p53 transcription activity [Bennett et al., 1998]. This resulted in diminished FasL-induced bystander cytotoxicity in these target cells. Though therapeutic strategies capable of modulating death receptor signaling pathways in combination with anticancer agents are only in its infancy, however, the recent increase of 5-FU-based chemotherapy in breast cancer, gives clinical relevance to our work, as it is very supportive and giving all the reasons for the participation of gap junction-mediated intracellular communication in the transmission of damage signals from alpha-particle irradiated to nonirradiated cells. Proc Natl Acad Sci USA 98:475-478.


REFERENCES

Albanese J, Dainiak N. 2000. Ionizing radiation alters Fas antigen ligand at the cell surface and on exfoliated plasma membrane-derived vesicles: Implications for


