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Influence of p53 and caspase 3 activity on cell death and senescence in response to methotrexate in the breast tumor cell

Deepali K. Hattangadi[a], Gerald A. DeMasters[a], Teneille D. Walker[a], Kara R. Jones[a], Xu Di[a], Irene F. Newsham[b], David A. Gewirtz[a,*]

[a]*Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298, USA*
[b]*David and Doreen Hermelin Laboratory of Molecular Oncogenetics, Department of Neurosurgery and Hermelin Brain Tumor Center, Henry Ford Hospital, Detroit, MI 48202, USA*

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Abstract

The influence of p53 function and caspase 3 activity on the capacity of the antifolate, methotrexate, to promote senescence arrest and apoptotic cell death was investigated in breast tumor cells. In p53 wild-type, but caspase 3 deficient MCF-7 breast tumor cells, death of approximately 40% of the cell population was observed immediately after acute exposure to 10 μM methotrexate (the IC_{80} value for a 2 h drug exposure). There was no evidence of either DNA fragmentation, a sub G_0 population or morphological alterations indicative of apoptosis; however, PARP cleavage was detected. Cell death was succeeded by growth arrest for at least 72 h—where arrest was characterized by expression of the senescence marker, beta-galactosidase. The response to methotrexate in MCF-7/E6 cells with attenuated p53 function was also primarily growth arrest—but lacking characteristics of senescence. In contrast, MCF-7 cells which expressed caspase 3 demonstrated a gradual and continuous loss of cell viability and unequivocal morphological evidence of apoptosis. DNA fragmentation indicative of apoptosis was also detected after exposure to methotrexate in p53 mutant MDA-MB231 breast tumor cells which also express caspase 3. Methotrexate-induced both p53 and $p21^{waf1/cip1}$ in MCF-7 cells within 6 h; however, no significant DNA strand breakage was evident before 18 h, suggesting that the induction of p53 reflects a response to cellular stress other than DNA damage, such as nucleotide depletion. Overall, these studies suggest that the nature of the cellular response to methotrexate depends, in large part, on p53 and caspase function. p53 appears to be required for methotrexate-induced senescence, but not apoptosis, caspase 3 is required for DNA fragmentation and the morphological changes associated with apoptosis, while neither p53 nor caspase 3 are required for methotrexate-induced growth arrest. Furthermore, the senescence phenotype may occur in the absence of direct DNA damage.

Keywords: Breast tumor; Apoptosis; Senescence; Methotrexate; Caspase 3; p53

1. Introduction

The antifolate methotrexate is commonly used in combination chemotherapy with cyclophosphamide and 5-fluorouracil for the treatment of breast cancer [1]. The selectivity of methotrexate is thought to be related, in large part, to its metabolism to polyglutamate derivates, which has also been shown to occur in the breast tumor cell [2]. Methotrexate polyglutamates inhibit various enzymatic activities associated with both purine and pyrimidine synthesis including thymidylate synthetase [3] and AICAR transformylase [4], as well as the most prominent and well characterized target, dihydrofolate reductase [5]. Methotrexate is an S phase specific drug which generally produces growth arrest in the G1 and/or S phases of the cell cycle [6,7].

Methotrexate has been shown to promote cell death through apoptosis in a number of experimental systems including HL60 and Jurkat leukemic cells [8,9], fibrosarcoma cells [10], and Chinese hamster ovary cells [11]. However, apoptosis is not a uniform response in tumors of differing tissue origins. Exposure of HT 29 colon cancer cells [12] or CEM leukemic cells [9] to methotrexate failed to elicit an apoptotic response. Apoptosis has been reported

* Corresponding author. Present address: Department of Medicine, Medical College of Virginia, Virginia Commonwealth University, 1101 East Marshall St., P.O. Box 980230, Richmond, VA 23298, USA. Tel.: +1 804 828 9523; fax: +1 804 828 8079.
E-mail address: gewirtz@hsc.vcu.edu (D.A. Gewirtz).

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in MCF-7 breast tumor cells after exposure to an inordinately high dose of methotrexate (in the range of 200–300 μM) by one group [13], but was not evident even at these elevated drug concentrations in a study by other investigators [14].

Resistance to methotrexate may occur through multiple mechanisms including reduced drug uptake [15], overexpression of one of the primary target enzymes dihydrofolate reductase [16], through interference with polyglutamate formation [17], and by enhanced drug efflux, mediated in part by the multidrug resistance protein 1 and the breast cancer resistance protein [18–20]. However, it is not clear whether failure to undergo apoptosis confers resistance to MTX in a manner similar to that often associated with DNA damaging agents [21,22].

Both growth arrest and apoptosis are associated with the induction of p53, particularly in response to DNA damage or other modes of cellular stress [21–25]. While the induction of both p53 and its downstream transactivation target, $p21^{waf1/cip1}$ by methotrexate have been observed [26], it is uncertain whether this induction reflects a DNA damage response. Methotrexate is thought to induce DNA strand breaks, but indirectly through the efforts of the cell to repair the misincorporation of dUTP into DNA [27], which occurs as a consequence of the inhibition of dUMP synthesis [28].

The current studies were designed to elucidate the nature of the response of breast tumor cells to methotrexate in terms of growth arrest and/or cell death. In view of recent findings that a form of senescence arrest may occur in response to antitumor drugs [29–32], this work examined senescence arrest in the context of p53 function. The frequent loss of caspase 3 function in clinical samples of breast cancer [33] suggested the importance of studying the response to methotrexate both in MCF-7 cells lacking functional caspase 3 [34] and the consequences of restoring caspase 3 function.

2. Materials and methods

2.1. Materials

RPMI 1640 medium with L-glutamine and trypsin-EDTA (1X; 0.05% trypsin, 0.53 mM EDTA-4 Na) was obtained from GIBCO BRL Life Technologies Inc. Penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin) and fetal bovine serum were obtained from Whittaker Bioproducts. Defined bovine calf serum was obtained from Hyclone Laboratories. Amethopterin (methotrexate), insulin from bovine pancreas, D-glucose, MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), Hoechst dye (bisbenzamide trihydrochloride), formaldehyde, acetic acid, bovine serum albumin (BSA) and dimethyl sulphoxide (DMSO) were obtained from Sigma Chemical. X-gal substrate was obtained from Gold BioTechnologies and Fluorescent dUTP dye from Roche Pharmaceuticals. Methotrexate was dissolved in water and maintained as a frozen stock solution at a concentration of 10 mM in PBS alkalinized with 1 N NaOH. Exposure to fluorescent light was minimized to prevent drug degradation.

2.2. Cell lines

MCF-7 breast tumor cells were obtained from the NCI Frederick Cancer Research Facility. MCF-7/NEO and MCF-7/E6 cells were developed as previously described [31]. E6-expressing cells expressed very low to undetectable levels of p53 protein and its downstream target $p21^{waf1/cip1}$ after acute adriamycin exposure [31]. MCF-cl.27/caspase 3-expressing cells (which will be referred to as MCF-7/caspase 3 cells) were generated by transfection of the pcDNA3-YAMA-x caspase 3 construct (a gift from M. Tewari) into MCF-cl.27 cells. The MCF-cl.27 cell line was generated from parental MCF-7 cells using the ecdysone inducible system (Invitrogen) and is inducible for expression of the tumor suppressor DAL-1/4.1B upon addition of the inducing agent muristerone [35]. Control experiments revealed no expression of DAL-1/4.1B protein in the absence of inducing agent in the generated MCF-cl.27/caspase 3-expressing cell line. Expression of caspase 3 was confirmed by Western blotting. MDA-MB-231 cells were obtained from ATCC (Bethesda, MD). All cell lines were maintained as monolayers in RPMI-1640 medium supplemented with 5% fetal bovine serum (Gibco), 5% bovine calf serum (Hyclone) and 0.5% penicillin/streptomycin (Gibco); and cultured at 37 °C in 5% CO_2.

2.3. Growth inhibition

The capacity of methotrexate to influence cell growth and viability was determined using the MTT tetrazolium dye assay [36]. Cells were plated in a 96-well microplate (Costar, Cambridge, MA) at a density of 4×10^4cells/ml, allowed to adhere to the surface overnight and incubated with various drug concentrations for either 2 or 24 h. Drug was removed by gentle inversion of the microplate, cells were washed twice with warm (37 °C), fresh complete medium, and incubated in drug-free medium for an additional 72 h prior to incubation for 4 h in an MTT solution (2 mg MTT/ml PBS) under low light conditions. The blue formazan product produced by mitochondrial succinate dehydrogenase activity was eluted from the cells after removal of the MTT solution and the addition of DMSO [37]. Absorbance at 490 nm was determined using an EL-800 auto microplate reader BIO-TEK instruments Inc., Burlington, VT.

2.4. Determination of viable cell number

The temporal response to methotrexate was assessed by monitoring viable cell number by trypan blue exclusion.

MCF-7 cells were plated at a density of 16×10^5 cells in 25 cm^2 T flasks (Costar, Cambridge, MA) and allowed to adhere overnight. Cells were incubated at 37 °C with 10 μM methotrexate for 2 h, washed twice with complete medium, and allowed to incubate in drug-free, complete medium. At the indicated times, the medium was aspirated, and the cells were washed twice with ice-cold PBS (pH 7.4), and viable cell counts determined in multiple flasks.

2.5. TUNEL assay for apoptosis

Apoptotic cell death was determined by the TUNEL assay [38] after treatment with 10 μM methotrexate for 2 h. Combined cytospins containing both adherent and non-adherent cells were fixed, and the fragmented DNA in cells undergoing apoptosis was detected using the In Situ Cell Death Detection Kit (Boehringer-Manheim) which end labels fragments with fluorescein dUTP via terminal transferase. Cells were washed, mounted in Vectashield and photographed using a Nikon fluorescent microscope.

2.6. Determination of DNA strand breaks by alkaline unwinding

DNA strand breaks were monitored using the alkaline unwinding procedure of Kanter and Schwartz [39]. MCF-7 cells in 75 cm^2 T flasks were exposed to 10 μM methotrexate for 2 h at 37 °C. Pooled aliquots of both adherent and nonadherent cells were subjected to alkaline unwinding as described in detail previously [40]. This assay is based on the differential binding and fluorescence of the indicator bisbenzamide trichloride (Hoechst 33258) to single strand and double strand DNA after a fixed period of alkaline denaturation. *F*-values which reflect the ratio of single to double stranded DNA were converted to radiation equivalence based on a standard curve generated using graded doses of radiation.

2.7. Cell cycle analysis

Cell cycle analysis was performed using standard propidium iodide staining and analyzed by flow cytometry [41]. Cells were drug-treated as above and at the indicated times, rinsed with cold PBS and incubated for 4 °C for 35–45 min with PIF (3.8 mM Na Citrate, 0.05 mg/ml propidium iodide, 0.1%Triton-X 100, 9 K units/ml of RNase B). Cells were scraped from the flask, combined with the nonadherent cells and stored at 4 °C. Just prior to flow analysis, each sample was vortexed and filtered through a 37 uM nylon mesh. Nuclei were analyzed with an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL) using the 488 nm line of an argon laser and standard optical emission filters. The resulting DNA distributions were analyzed for the proportion of cells in various stages of the cell cycle using Cytologic Software (Coulter Electronics).

2.8. Western analysis for protein expression

At the indicated times after drug exposure, cells were washed in PBS and lysed using 100–200 μl of lysis buffer containing protease inhibitors for 30 min on ice. Following the determination of protein concentrations by the Lowry method [42] 10 or 20 μg aliquots of cell protein were separated using 15% SDS–PAGE. Proteins were transferred onto a nitrocellulose membrane and blocked in TBS-tween buffer containing 5% non-fat dry milk. Antibodies to p53 and p21 were obtained from Pharmingen/Transduction Laboratories, San Diego, CA, and diluted 1:5000 and 1:500, respectively. Membranes were immunoblotted with the respective antibodies and incubated with horse radish peroxidase conjugated goat anti-mouse secondary antibody (KPL Laboratories, Gaithersburg, MD). Proteins were visualized using an enhanced chemiluminescence kit from PIERCE.

Blots were stripped by a 30 min incubation at 55 °C in a solution consisting of 62.5 mM Tris–HCl, pH6.8, 2% SDS and 100 mM beta-mercaptoethanol. The blots were reprobed with actin antibody at a dilution of 1:3000 and visualized after exposure to the secondary anti-rabbit antibody.

For determination of PARP cleavage, proteins were separated by electrophoresis in 8% SDS–PAGE gels. Membranes were immunoblotted with the PARP antibody (Biomole, Plymouth Meeting, PA) diluted 1:3000 and horse radish peroxidase labeled goat (anti-mouse) secondary antibody. The intact (116 kDa) and cleaved (85 kDa) forms of PARP were detected.

2.9. Beta-galactosidase assay for senescence

Cells were seeded in 6-well plates, protected from light and exposed to 10 μM methotrexate for 2 h, as described above and processed as described previously [31]. At the indicated times, cells were washed twice with PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS, and washed twice in PBS. Cells were stained overnight in X-gal staining solution [1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6), 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$] [43]. Stain was washed off the following day, PBS was added to the wells and staining was viewed under an Olympus® America fluorescence microscope.

3. Results

3.1. Determination of sensitivity to methotrexate

Sensitivity to methotrexate was determined in MCF-7 breast tumor cells, MCF-7/E6 cells with attenuated p53 function, and MCF-7/caspase 3 cells utilizing a standard MTT dye assay after a 2 h drug exposure. Fig. 1(A) shows

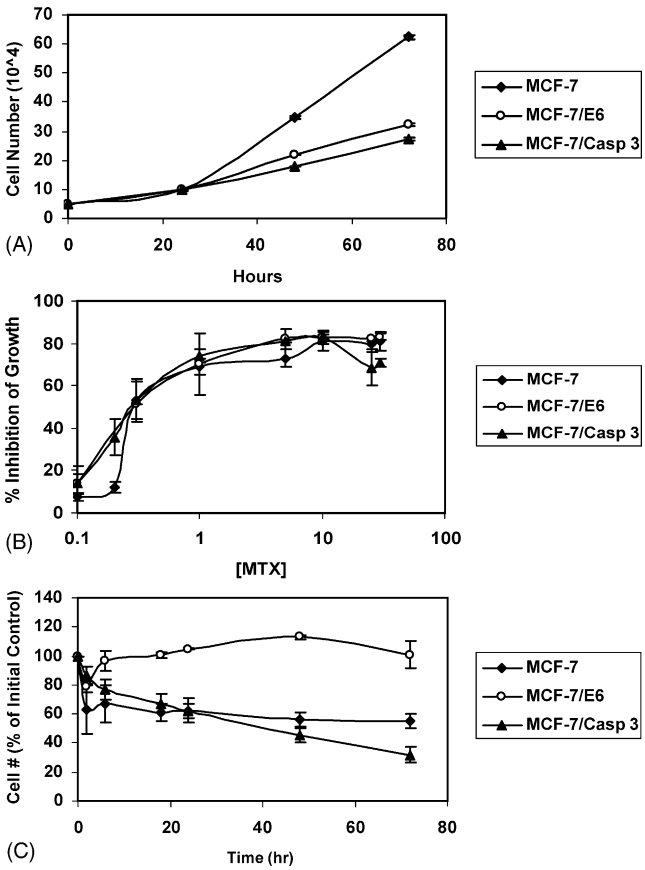

Fig. 1. (A) Growth of MCF-7, MCF-7/E6 and MCF-7/caspase 3 cells. Cells were seeded in 6-well plates at a density of 5×10^4 cells/plate. Cell number was assessed in triplicate plates and similar results were generated in a duplicate experiments. Standard errors are encompassed within the symbols. (B) Determination of sensitivity to methotrexate in MCF-7, MCF7/E6 and MCF-7/caspase 3 cells by the MTT day assay. Viable cell number was compared in cells exposed to methotrexate for 2 h and in untreated cells 3 days after drug exposure. Values represent means ± standard errors (or range) for seven replicate experiments (MCF-7 cells), four experiments (MCF-7/E6 cells) and two experiments (MCF-7/caspase 3 cells). (C) Temporal response to methotrexate in MCF-7, MCF-7/E6 and MCF-7/caspase 3 cells. Cells were exposed to 10 μM methotrexate for 2 h and viable cell number was monitored at the indicated times after drug exposure. Values represent means ± standard errors (or range) for three replicate experiments for MCF-7 and MCF-7/E6 cells and two experiments for MCF-7/caspase 3 cells.

that growth rates of the MCF-7/E6 cells and the MCF-7/caspase 3 cells were reduced as compared to MCF-7 cells. As shown in Fig. 1(B), IC_{50} values for all three cell lines converged at approximately 0.3 μM, with 80% growth inhibition evident at a drug concentration of approximately 10 μM[1]. The IC_{50} for a 24 h exposure of MCF-7 cells to methotrexate was approximately 0.2 μM (not shown).

3.2. Temporal response to methotrexate

We chose to utilize the 10 μM concentration of methotrexate for subsequent experiments as clinical pharmacokinetic studies have demonstrated serum levels of methotrexate in this concentration range [44,45]. Fig. 1(C) shows the temporal response of MCF-7, MCF-7/E6, and MCF-7/caspase 3 cells to a 2-h exposure to 10 μM methotrexate. In MCF-7 cells, an initial and quite rapid loss (i.e. cell death) of approximately 40% of the cell population was succeeded by growth arrest which lasted for at least 72 h. MCF-7/E6 cells quickly recovered from a small initial decline in cell number (approximately 15–20%) that was succeeded by a similar growth arrest response. MCF-7/caspase 3 cells demonstrated a gradual and continuous decline in viable cell number over the 72 h time course of the study resulting in loss of approximately 70% of the cell population by 72 h after methotrexate exposure[2].

3.3. Apoptosis induction after methotrexate

In order to determine whether the initial cell death observed in MCF-7 cells exposed to methotrexate might be due to apoptosis, DNA fragmentation was assessed using TUNEL analysis and, as indicated below, by cell cycle analysis. Fig. 2(A) indicates that few fluorescent MCF-7 cells indicative of apoptosis were detected at 18 h after exposure to methotrexate. Furthermore, there was no indication of apoptotic morphology as the cells remained smooth and rounded. Similarly, there was no evidence of apoptosis at 2 and 6 h (data not shown) despite the fact that a marked decline in viable cell number was evident within the first 6 h of exposure of MCF-7 cells to methotrexate.

Apoptosis was also assessed by the TUNEL assay in MCF-7/E6 cells which are deficient in functional p53 as well as lacking caspase 3. Fig. 2(A) indicates that no apoptosis was evident in these cells over the course of 18 h (similar data was generated for 2 and 6 h post methotrexate exposure, not shown). This was expected from the minimal cell death observed in MCF-7/E6 cells after exposure to methotrexate.

In order to substantiate the absence of apoptosis in MCF-7 cells, cell cycle distribution was determined at various times after exposure to 10 μM methotrexate by PI staining and flow cytometry. Fig. 2(C) confirms the lack of apoptosis induction in MCF-7 cells by methotrexate, as few cells were detected in the sub-G_0/G_1 population. These experiments also show a pronounced suppression of the G_2/M phase of the cell cycle with an accumulation of cells primarily in the G_1 phase by 24 h post drug exposure.

As MCF-7 cells do not express functional caspase 3 [34], it appeared possible that the absence of this executioner caspase might prevent the morphological changes and

[1] Sensitivity to methotrexate was similar in MCF-7 cells transfected with an empty (neo) vector control (not shown).

[2] The small differences in absolute cell number shown in Fig. 1(C) for MCF-7, MCF-7/E6 and MCF-7/caspase 3 cells would not be detected as differential sensitivity by the MTT assay in Fig. 1(B).The MTT assay measures the difference in viable cell number between control and drug-treated cells after 3 days, approximately four doubling times for the control cells (which are not shown). The data in Fig. 1(C) reflect cell number in relation to initial control values.

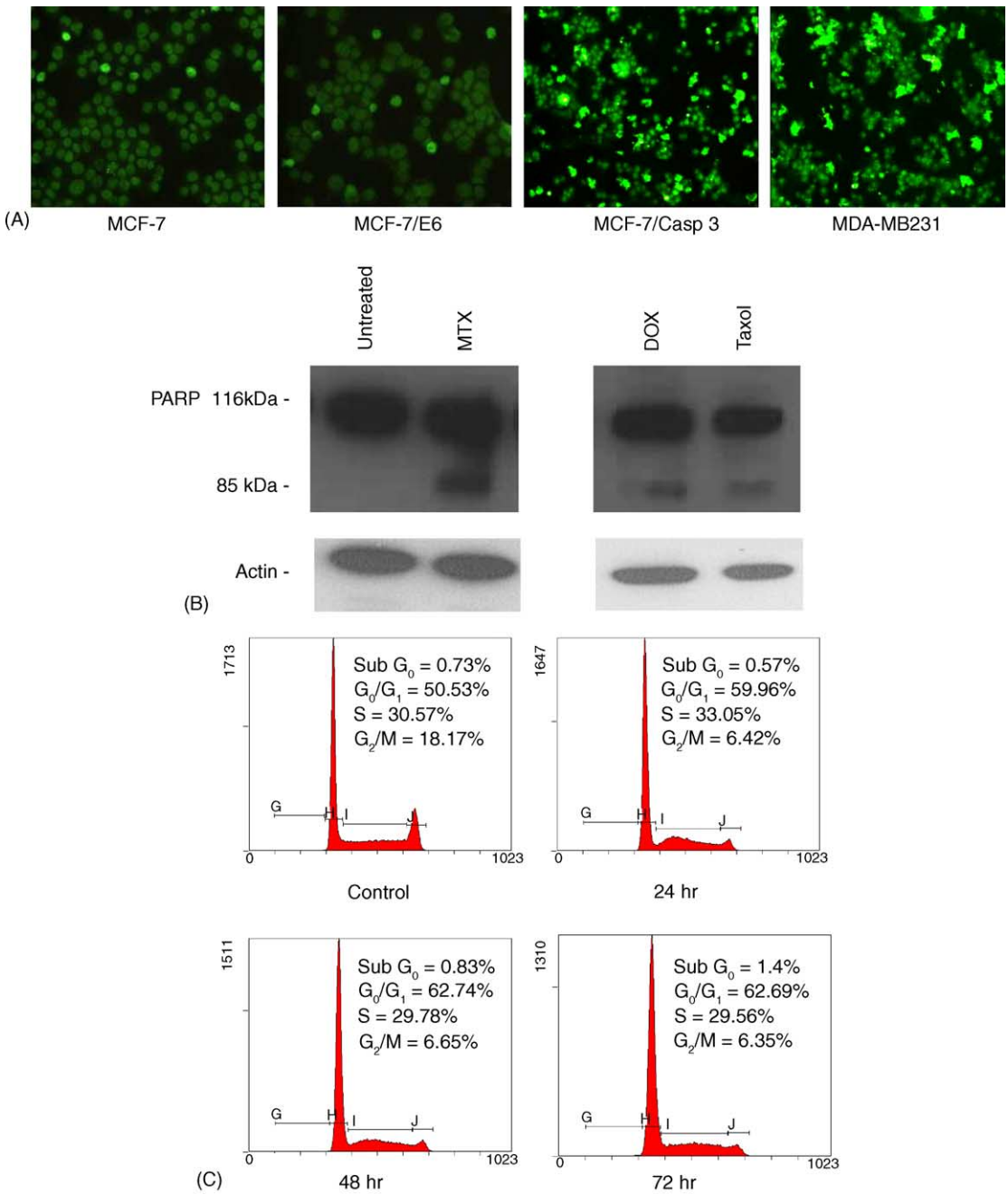

Fig. 2. (A) Assessment of apoptosis by the TUNEL assay in MCF-7, MCF-7/E6 and MCF-7/caspase 3 and MDA-MB231 cells. Cells were exposed to 10 μM methotrexate for 2 h and both adherent and nonadherent cells were combined for analysis of apoptosis. (B) PARP cleavage in MCF-7 cells exposed to either 10 μM methotrexate for 2 h, 5 μM taxol for 3 days or 17 μM doxorubicin for 2 days. (C) Cell cycle distribution in MCF-7 cells after exposure to methotrexate. Distribution of cells within the different phases of the cell cycle was determined by PI staining after exposure to 10 μM methotrexate for 2 h. This figure is representative of two separate experiments.

DNA fragmentation associated with apoptotic cell death. We therefore assessed cleavage of PARP, an alternative marker of the apoptotic signaling pathway [46]. Fig. 2(B) indicates that PARP cleavage was clearly evident in MCF-7 cells exposed to methotrexate (as well as to high concentrations of doxorubicin and taxol which were used as positive controls).

Fig. 2(A) provides further evidence that the morphological changes and DNA fragmentation which tend to define classical apoptosis are dependent on the presence of caspase 3. The studies in MCF-7/caspase 3 cells presented in Fig. 2(A) show unequivocal evidence of alterations in cell morphology such as cell shrinkage and irregularity (as well as DNA fragmentation indicated by nuclear fluorescence) in MCF-7/caspase 3 cells at 18 h after exposure to methotrexate. Apoptotic cells were detected as early as 2 h after drug exposure (not shown). This finding was unexpected, as we and others have determined that exogenous expres-

sion of caspase 3 is not sufficient for induction of apoptosis in MCF-7 cells in response to radiation [47].

To determine whether functional p53 was required for the induction of classical apoptotic cell death in the breast tumor cell, DNA fragmentation and cell morphology were also assessed in non-isogenic p53 mutant MDA-MB231 cells which express caspase 3. Again, Fig. 2(A) provides clear evidence for apoptosis in this cell line as well. Taken together, the data presented in Fig. 2 suggest that methotrexate can induce select elements of apoptosis such as PARP cleavage even in breast tumor cells which lack caspase 3. However, the capacity of methotrexate to promote DNA fragmentation and morphological indicators of apoptosis is clearly dependent on caspase 3, but can occur in the absence of functional p53.

3.4. Senescence induction after methotrexate

Studies have indicated that the predominant response of various solid tumor cell lines with functional p53 to both chemotherapeutic drugs and ionizing radiation is often a senescence-like growth arrest [29–32,66]. There is further recent evidence that apoptosis and senescence may represent reciprocally regulated and mutually exclusive responses to drug treatment [48]. To determine whether the growth arrest observed in MCF-7 cells after methotrexate was characteristic of senescence, cells were stained with beta-galactosidase at selected times after drug exposure. Fig. 3 demonstrates extensive and widespread beta-galactosidase staining in MCF-7 cells at 96 h after exposure to 10 μM methotrexate. (In a separate study, extensive beta-galactosidase staining was also evident within 72 h, not shown). In contrast, minimal beta-galactosidase staining was detected in the MCF-7/E6 cells (Fig. 3); the senescence response was also evident in MCF-7/neo cells (not shown). Finally, a small amount of beta-galactosidase staining was also detected in the MCF-7/caspase 3 cells after 96 h. These findings are consistent with previous work suggesting the involvement of p53 in the accelerated senescence response to antitumor drugs and radiation in the tumor cell [29–32].

3.5. Induction of p53 and $p21^{waf1/cip1}$ and DNA strand breaks by methotrexate

Induction of p53 and one of its downsream targets, $p21^{waf1/cip1}$, are involved in both arrest at the G_1 to S transition as well as accelerated senescence [24,25,30]. Although p53 induction is generally associated with DNA damaging agents, increases in levels of p53 may also reflect generalized cellular stress resulting from nucleotide pool depletion [49,50]. As shown in Fig. 4(A), acute exposure to methotrexate results in elevations in both p53 and $p21^{waf1/cip1}$ proteins within 6 h, and that p53 and $p21^{waf1/cip1}$ are maintained at elevated levels for at least 18 h.

Interestingly, Fig. 4(B) indicates that no DNA strand breaks (i.e. fragmentation exceeding baseline levels) could be detected in MCF-7 cells over the course of 2–6 h after exposure to methotrexate using the alkaline unwinding assay. A small degree of DNA fragmentation and/or strand breakage was evident between 18 and 48 h (approximately 80 rad equivalents after subtraction of background values), while the extent of DNA damage was increased to 200 rad equivalents (again, after subtraction of baseline levels) by 72 h.

4. Discussion

Reports in the literature have provided conflicting data relating to methotrexate sensitivity in the breast tumor cell with IC_{50} values reported to range from nanomolar to high micromolar concentrations [51–55]. We find an IC_{50} value of approximately 300 nM with a 2 h drug exposure and 200 nM with a 24 h exposure, which agrees with the reports of Volk et al. [54] and Laque-Ruperez et al. [55].

The assessment of the temporal response to methotrexate revealed that approximately 40% of the MCF-7 cell population lost viability within 12–18 h, while the greater portion of the cell population demonstrated growth arrest. The heterogeneity of the response may reflect enhanced susceptibility of the S phase population to cell killing by methotrexate. Cell cycle analysis indicated that approximately 30% of the logarithmically growing cell population is in the S phase.

Despite the finding of PARP cleavage, there was minimal evidence of apoptosis in either the MCF-7 or the MCF-7/E6 cells using three separate and complementary assays for DNA fragmentation and morphology changes in the MCF-7 cells. In contrast, there was clear evidence of classical apoptosis in the MCF-7/caspase 3 cells and the MDA-MB231 cells with mutant p53 but functional caspase 3. These findings extend earlier work which has shown that caspase 3 activity is required for promotion of classical apoptosis in response to agents such as TNF-alpha and staurosporine [56]. In this context, our previous work has demonstrated that the lack of caspase 3 does not prevent apoptosis in MCF-7 cells primed by exposure to vitamin D_3 analogs prior to being challenged with radiation or adriamycin [57,58].

The current studies further indicate that p53 is not required for apoptosis after methotrexate. Interestingly, the differential response to methotrexate in the absence and presence of caspase 3 appears to be agent specific, as no apoptosis is evident in response to ionizing radiation in caspase 3-expressing MCF-7 cells [47]. It is furthermore of interest that studies utilizing drugs such as etoposide and doxorubicin demonstrate essentially equivalent cell killing in the absence and presence of caspase 3 expression [59], despite the fact that restoration of caspase 3 promotes sensitivity to apoptosis. While these observations might

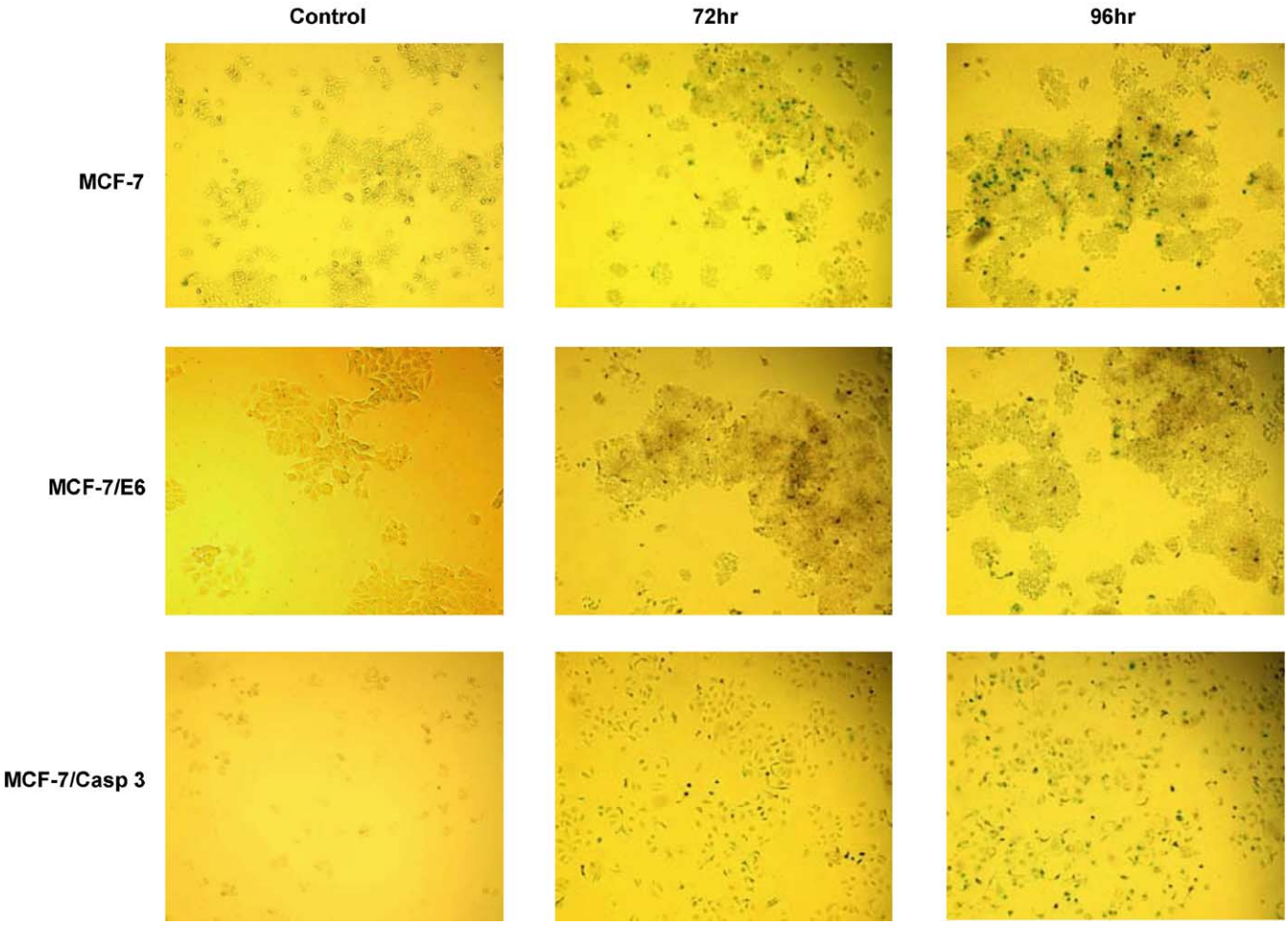

Fig. 3. Beta-galactosidase staining as an indicator of senescence arrest in MCF-7, MCF-7/E6 and MCF-7/caspase 3cells. Cells were exposed to 10 μM methotrexate for 2 h and beta-galactosidase staining was evaluated over a time frame of 96 h. These findings were reproduced multiple times.

suggest that promotion of apoptosis is of little consequence to the cellular response to methotrexate, this is clearly not the case in the current studies, where the absence of caspase 3 leads to widespread senescence arrest, while restoration of caspase 3 is permissive for a prolonged and apparently continuous cell death response. This work parallels recent studies where interference with apoptosis using caspase inhibitors led to senescence arrest [48].

The growth arrest that succeeds cell death in MCF-7 cells involves a marked reduction of the G_2/M population, with accumulation of cells primarily in G_1. This observation is consistent with previous reports of methotrexate action in other tumor cell lines [6,7]. What appears to be quite unique and previously unreported is the finding of the expression of beta-galactosidase, a marker of replicative senescence [43], which may be related to the induction of p53 and $p21^{af1/cip1}$ [29–32]. The absence of a senescence response to methotrexate in the MCF-7/E6 cells lacking functional p53 (which nevertheless do arrest growth) lends further support to this paradigm. As in our previous studies with adriamycin and irradiation [31], the senescence arrest response to methotrexate appears to be independent of p16, as p16 is not expressed in MCF-7 cells [60,61]. The findings relating to p53 and caspase 3, senescence, growth arrest, and apoptosis are summarized in Table 1.

Induction of p53 and $p21^{waf1/cip1}$do not appear to be related to DNA strand breakage by methotrexate as the changes in p53 and $p21^{waf1/cip1}$ protein levels precede the breaks detected by alkaline unwinding. The DNA breaks which became evident at later times are likely to reflect damage from the cell attempting to repair misincorporation of dUTP [27,28]. These findings add to the growing body of literature suggesting that senescence-like growth arrest (or accelerated senescence) in tumor cells is likely to be a generalized stress response that is mediated by p53 and $p21^{waf1/cip1}$ [29–32]. The stress detected by the cell may reflect nucleotide depletion, which has been shown to occur within hours after exposure to methotrexate [50].

The finding of apparently similar sensitivity to methotrexate in MCF-7 cells with functional p53 which senesce, MCF-7/E6 cells with attenuated p53 which arrest without undergoing apoptosis or senescence, and MCF-7/caspase 3 cells which undergo apoptosis suggests that the manner in which a cell dies does not influence drug sensitivity. This

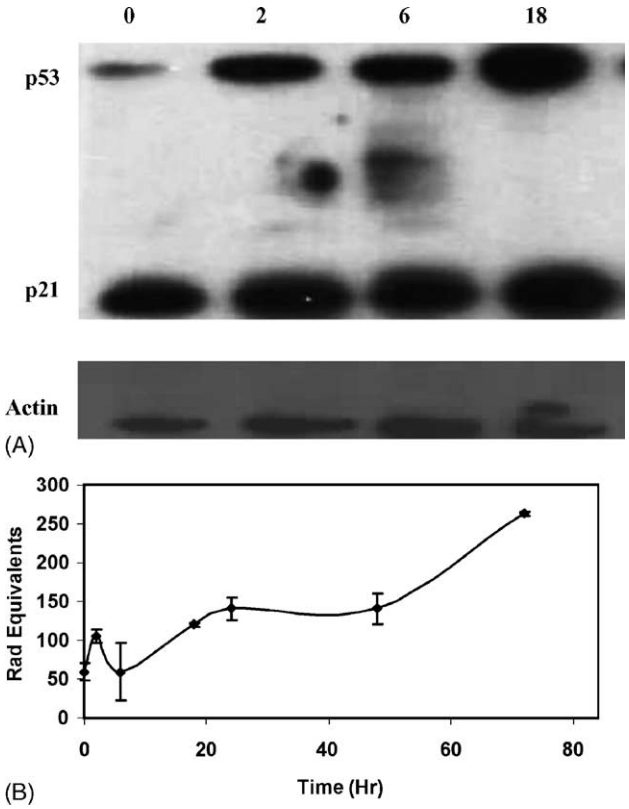

Fig. 4. (A) Induction of p53 and $p21^{waf1/cip1}$ by methotrexate in MCF-7 breast tumor cells. MCF-7 cells were exposed to 10 μM methotrexate for 2 h and induction of p53 and $p21^{waf1/cip1}$ was monitored by Western blotting. Actin was utilized as a loading control. (B) Induction of DNA strand breaks/DNA fragmentation in MCF-7 cells as assessed by alkaline unwinding. Cells were treated with 10 μM MTX for 2 h and DNA strand breakage was evaluated at indicated time points after drug exposure. Values represent mean values ± range for two experiments.

Table 1
Summary of p53 and caspase 3 dependence of growth arrest, apoptosis and senescence in response to methotrxate in MCF-7 breast tumor cells

	Growth arrest	Apoptosis	Senescence
p53	Independent	Independent	Dependent
Caspase 3	Independent	Dependent	Independent

may in fact be true when assessing the initial response. However, there is accumulating evidence that drug-induced senescence arrest may be reversible [32,62] while apoptosis is unequivocally an irreversible phenomenon. Studies have shown recovery of proliferative activity after exposure to methotrexate in MCF-7 and L1210 leukemic cells[3] [63,64] and in vivo [65]. The tumor growth delay that is observed in studies of drug or radiation treatment of tumor cell xenografts may, in fact, reflect senescence arrest followed by recovery. Since it is likely that drug-induced apoptosis is antagonistic to any resurgence of proliferative capacity, differing modes of cell death and growth arrest could have a substantive influence on breast tumor cell regrowth. This possibility will be the focus of future research efforts.

[3] It has not been established whether the response to methotrexate in leukemic cells may reflect, in part, senescence arrest.

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