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Apoptosis, autophagy, accelerated senescence and reactive oxygen in the response of human breast tumor cells to Adriamycin

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ABSTRACT

Although the primary response to Adriamycin (doxorubicin) in p53 mutant MDA-MB231 and p53 null MCF-7/E6 breast tumor cells is apoptotic cell death, the residual surviving population appears to be in a state of senescence, based on cell morphology, beta galactosidase staining, induction of $p21^{waf1/cip1}$ and down regulation of cdc2/cdk1. Suppression of apoptosis in MDA-MB231 and MCF-7/E6 cells treated with Adriamycin using the broad spectrum caspase inhibitor, zvad-Fmk, results in substantial induction of autophagy. Overall sensitivity to Adriamycin, measured by clonogenic survival, is not altered in the cells undergoing autophagy, consistent with autophagy contributing to cell death in response to Adriamycin. The free radical scavengers, glutathione and N-acetyl cysteine attenuate the accelerated senescence response to Adriamycin in MCF-7 cells as well as in MDA-MB231 and MCF-7/E6 cells, but protect primarily the MCF-7 cells, indicating that reactive oxygen is unlikely to be directly responsible for Adriamycin toxicity in breast tumor cells. Expression of caspase 3 or induced expression of c-myc in MCF-7 cells fails to abrogate accelerated senescence induced by Adriamycin. Taken together, these studies suggest that accelerated senescence induced by Adriamycin is similar in cells with wild type p53 and in cells lacking functional p53 with regard to the upregulation of $p21^{waf1/cip1}$, down regulation of cdc2 and the involvement of reactive oxygen species. Furthermore, accelerated senescence, autophagy and apoptosis all appear to be effective in suppressing self-renewal capacity in breast tumor cells exposed to Adriamycin.

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1. Introduction

It is becoming increasingly apparent that tumor cells have the capacity to respond to chemotherapy and radiation through multiple growth arrest and cell death pathways [1,2]. Cell death in leukemia and lymphoma derived tumor cells frequently occurs through apoptosis [3,4], while mitotic catastrophe is a well established response to ionizing radiation [5]; in addition,

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Abbreviations: ADR, Adriamycin (doxorubicin); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; GSH, glutathione; NAC, N-acetyl cysteine.

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autophagy or type II apoptosis is recognized as having the potential to contribute to cell killing in addition to its known cytoprotective function associated with nutrient deprivation [6,7]. Self-renewal capacity in the tumor cell can also be abolished through accelerated or premature senescence, a form of growth arrest that has been observed primarily, although not exclusively, after exposure to ionizing radiation or drugs that induce DNA damage [8,9]. Like replicative senescence, cells undergoing accelerated senescence are characterized by enlargement, flattening, granulation and expression of a pH 6.0 dependent beta galactosidase activity [8–10]. Accelerated or premature senescence has also been identified in tumor cell xenografts exposed to chemotherapeutic agents [2], has been shown to mediate tumor regression [11,12], in part through the involvement of the immune system, and has been reported in clinical tumor samples in patients receiving chemotherapy [13,14].

The signaling pathway that promotes accelerated senescence overlaps, in large part, with that for conventional growth arrest in terms of the induction of p53 [15] and the cyclin dependent kinase inhibitory protein, $p21^{waf1/cip1}$ [15] as well as down regulation of cdc2/cdk1 [9,13,16,17]; however, there is also evidence for accelerated senescence that is independent of p53 [13,18]. While the induction of senescence does not appear to require functional p16 [18–21], p16 may be critical for maintenance of the senescence-arrested state [22,23]. Suppression of c-myc has been shown to promote senescence [24–26], an observation which may be related to the capacity of both p53 and $p21^{waf1/cip1}$ to suppress transcription of c-myc [27,28]. Finally, mitochondrial reactive oxygen generated downstream of $p21^{waf1/cip1}$ has been implicated in replicative senescence [29], while the role of reactive oxygen, if any, in accelerated senescence awaits definition.

In view of the fact that tumor cells can respond to stresses such as radiation and chemotherapeutic drugs through alternative cell death pathways when the ''primary'' pathway is compromised or attenuated [30–35], we were interested in assessing whether multiple mode(s) of cell death (and/or growth arrest) mediate the response to the chemotherapeutic drug, Adriamycin, in breast tumor cells that have been shown to be highly apoptosis competent (cells mutant or null in p53 as well as p53 wild type MCF-7 cells expressing caspase 3 [20,33]). We also further addressed the signaling pathways involved in the accelerated senescence response to Adriamycin, focusing on the induction of $p21^{waf1/cip1}$, the down regulation of cdc2 and c-myc and the potential contribution of reactive oxygen species.

2. Materials and methods

2.1. Materials

RPMI 1640 medium with L-glutamine, trypsin-EDTA (1×; 0.05% trypsin, 0.53 mM EDTA-4 Na), penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin), and fetal bovine serum were obtained from Invitrogen (Eugene, OR). Defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). Reagents used for the TUNEL assay (terminal transferase, reaction buffer, and Fluorescein-dUTP) were purchased from Roche Diagnostics Corporation (Germany). X-gal was obtained from Gold Biotechnology (St. Louis, MO). The following materials were obtained from Sigma Chemical (St. Louis, MO): formaldehyde, acetic acid, albumin bovine (BSA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), monodansylcadaverine (MDC), N-acetyl-L-cysteine (NAC), reduced glutathione (GSH), 6-diamidino-2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO). Acridine orange was purchased from Molecular Probes (Eugene, OR). zVAD-fmk was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Adriamycin was obtained from Sigma Chemical Company, St. Louis, MO, reconstituted in molecular biology grade water, and stored as aliquots at −20 °C until dilution in culture media for treatments. Antibodies for p53 and $p21^{WAF-1}$ were purchased from Signal Transduction Laboratories), cdc-2 from Santa Cruz Biotechnology, anti-mouse IgG from KPL Inc. and β-actin from Sigma Chemical Company. Reporter constructs for the luciferase assay were purchased from Addgene Inc. (Cambridge, MA). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI).

2.2. Cell culture and treatment regimens

The MCF-7 breast tumor cell line was obtained from the NCI Frederick Cancer Research Facility. The isogenic cell line, MCF-7/E6, was established by stable retroviral infection as described previously [20]. MCF-7/35im cells with doxycycline-inducable myc were established as described previously [36]. The MCF-7 cells expressing caspase 3 were described in previous studies [33]. Cells were maintained as monolayer cultures in RPMI 1640 media supplemented with glutamine (0.292 mg/ml), penicillin/streptomycin (0.5 ml/100 ml media), and 10% fetal bovine serum. Cells were cultured at 37 °C in 5% CO_2 and 100% humidity. 24 h after plating, cells were exposed to either 0.75 μM or 1 μM Adriamycin for 2 h. Cells were washed free of drug and cultured in fresh media for the subsequent period of the experimental protocols.

2.3. Effects on GASH and NAC on sensitivity to Adriamycin by the MTT assay

To determine the effects of GSH or NAC on sensitivity to Adriamycin in MCF-7, MDA-MB231 and MCF-7/E6 cells, cells were seeded in triplicate wells at 8000 cells per well of a 96-well cluster plate and were treated with 1 μM of Adriamycin for 2 h in the presence or absence of 20 mM GSH or 20 mM NAC, followed by removal of the drugs and washing of the cells. At 72 h post-drug exposure, cell viability was assessed using a standard MTT assay. This involved adding 100 μL of 2 μg/mL MTT per well, incubating in the dark for 3 h, carefully removing the MTT, and then adding 100 μL DMSO per well. Absorbance was measured at 490 nm with an EL800 Universal Microplate Reader (Bio-Tek Instruments Inc.).

2.4. Beta-galactosidase staining

Senescence-associated (SA) beta-galactosidase histochemical staining in the MDA-MB231 and MCF-7/E6 cells was performed as described previously [10,20] after exposure to 0.75 μM ADR. Cells were washed twice with PBS and fixed with 2%

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