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Thiol Redox Modulation of Doxorubicin Mediated Cytotoxicity in Cultured AIDS-Related Kaposi's Sarcoma Cells

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Abstract The chemotherapeutic, doxorubicin, is currently used empirically in the treatment of AIDS- related Kaposi's sarcoma (AIDS-KS). Although often employed in a chemotherapeutic cocktail (doxorubicin, bleomycin, vincristine) single-agent therapy has recently been attempted with liposome encapsulated doxorubicin. Although doxorubicin's mechanism of action against AIDS-KS is unknown, we hypothesized that doxorubicin's ability to undergo redox cycling is associated with its clinical efficacy. The current study was conducted to investigate the effects of doxorubicin on selected xenobiotic-associated biochemical responses of three cellular populations: KS lesional cells, nonlesional cells from the KS donors, and fibroblasts obtained from HIV^- aged matched men. Our results show that during doxorubicin challenge, there are strong positive correlations between cellular glutathione (GSH) levels and viability ($r = 0.94$), NADPH levels and viability ($r = 0.93$), and GSH and NADPH levels ($r = 0.93$), and demonstrate that as a consequence of their abilities to maintain cellular thiol redox pools HIV^- donor cells are significantly less susceptible to doxorubicin's cytotoxic effects relative to AIDS-KS cells. Additional studies further supported the contribution of reduced thiols in mediating doxorubicin tolerance. While pretreatment with the GSH precursor, N-acetylcysteine was cytoprotective for all cell groups during doxorubicin challenge, GSH depletion markedly enhanced doxorubicin's cytotoxic effects. Studies to investigate the effects of a hydroxyl scavenger and iron chelator during doxorubicin challenge showed moderate cytoprotection in the AIDS-KS cells but deleterious effects in the HIV^- control cells. Inactivation of the longer lived membrane generated ROI in the cytoprotective deficient AIDS-KS cells, as well as an impairment of endogenous defenses in the HIV^- donor control cells, may account for these scavenger and chelator associated findings. In summary, our findings show that doxorubicin mediates, at least in part, its AIDS-KS cellular cytotoxic effects by a redox related mechanism, and provides a biochemical rationale for doxorubicin's clinical efficacy in AIDS-KS treatment. J. Cell. Biochem. 73:259–277, 1999. © 1999 Wiley-Liss, Inc.

Key words: glutathione; pyridine nucleotides; oxidant stress; clinical management; chemotherapeutics

AIDS-related Kaposi's sarcoma (AIDS-KS) is both the most common HIV associated malignancy, and an AIDS-defining disease [Kriegel and Friedman-Kein, 1990]. While the precise pathogenesis of AIDS-KS remains speculative, both clinical and experimental evidence suggest that AIDS-KS has a multifactorial etiology, with factors such as latent virus induction, sustained inflammation, and subsequent elevations in cytokines contributing to disease progression [Jacobson and Armenian, 1995; Miles, 1994; Moore et al., 1996]. Notably, AIDS-KS cells are excellent producers of autologous growth promoting cytokines [Ensoli et al., 1989, 1992; Miles et al., 1990]. Therefore, cytokine expression by incipient AIDS-KS lesions not only facilitates local cellular growth, but also promotes the development of new tumor foci [Ensoli et al., 1989, 1992; Miles et al., 1990].

Currently, the treatments for AIDS-KS are primarily palliative (surgical excision, radiation, chemotherapy), and all are associated with significant morbidity [Northfelt, 1994; Tappero et al., 1993]. For individuals with disseminated disease, combination agent systemic chemother-

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apy (doxorubicin, bleomycin, and vincristine) has been employed, resulting in bone marrow suppression which exacerbates the underlying immune deficit [Northfelt, 1994; Tappero et al., 1993]. Recently, liposome encapsulated doxorubicin is being administered intravenously in an effort to enhance drug concentrations at AIDS-KS lesional sites [Coukell and Spencer, 1997]. Apparently, doxorubicin was selected for the liposome encapsulation delivery system on the basis of its pharmacological properties as well as the clinical evidence suggesting doxorubicin's efficacy in AIDS-KS treatment [Coukell and Spencer, 1997].

Doxorubicin can elicit its cytotoxic effects via at least two routes, which are contingent upon varied factors including drug concentration and cellular location [Cutts et al., 1996; Dorochow, 1983; Lui, 1989; Taatjes et al., 1997]. At relatively low concentrations, doxorubicin accumulates primarily in the cellular nucleus, where it intercalates with the cellular DNA and subsequently perturbs the function of a key DNA synthesizing enzyme, topoisomerase II. In some cells, doxorubicin is also speculated to undergo redox cycling via reduction to the semiquinone by NADH or NADPH reductases. Further, because many nicotinamide nucleotide-dependent reductases are located in either the mitochondrion or microsomes, doxorubicin administration can result in the increased generation of deleterious reactive oxygen intermediates (ROI) at vital sites of ongoing cellular oxidative metabolism. Previous in vitro studies to evaluate the mechanism of doxorubicin mediated cellular cytotoxicity have provided variable results, and have shown that in some cellular strains doxorubicin does not appear to induce toxicity via a redox dependent route [DeGraff et al., 1994; Fry et al., 1991; Mestdagh et al., 1994]. However, cumulatively these prior studies have identified several parameters which appear to closely correlate with cellular susceptibility to doxorubicin including: tissue or lesion of cellular origin, cellular Phase II enzyme expression, and cellular capacity to promptly upregulate cellular glutathione (GSH) levels subsequent to doxorubicin challenge. [Lee et al., 1989; Liu and Kehrer, 1996; Paranka and Dorr, 1994; Russo and Mitchell, 1989].

Currently, doxorubicin is used empirically in the management of AIDS-KS. Further, to our knowledge, no investigations have yet been conducted to elucidate the mechanism of action of doxorubicin in AIDS-KS cells. Although clarification of how doxorubicin mediates cytotoxicity in AIDS-KS cells would be of scientific interest, more essentially, an understanding of doxorubicin's mechanism of action would facilitate the development of novel agents even more selective in their antitumor effects.

Several studies from our laboratory have shown that AIDS-KS cells have an impaired capacity to biochemically respond to oxidant stress at both the bioenergetic and cytoprotective enzyme levels. [Mallery et al., 1994, 1995] Further, our laboratory has recently shown that as a consequence of an inherent perturbation in their cellular thiol redox status, AIDS-KS cells show an accentuated response to tumor necrosis factor α, a cytokine which employs reactive oxygen intermediates (ROI) in its signal transduction pathway [Mallery et al., 1998]. Collectively our data, which show that ROI persist longer in AIDS-KS cells, suggests that AIDS-KS cells should therefore demonstrate enhanced susceptibility to redox active agents such as doxorubicin [Mallery et al., 1994, 1995, 1998]. This current study was conducted to investigate the effects of doxorubicin on the cellular thiol redox bioenergetic status of three defined cellular populations, i.e., KS lesional cells, nonlesional cells from the KS donors, and fibroblasts obtained from HIV^- aged matched men. The results presented in this paper demonstrate that doxorubicin tolerance is correlated with cellular capacity to maintain a specific pool of reducing equivalents and oxidant scavengers, i.e., NADPH and GSH, and show that as a consequence of their altered thiol redox status AIDS-KS cells are significantly more susceptible to doxorubicin. Our findings also suggest that doxorubicin mediates, at least in part, its AIDS-KS cellular cytotoxic effects by a redox-related mechanism.

MATERIALS AND METHODS

Overall Experimental Approach

These studies were designed to provide a profile of specific cellular responses (viability, GSH levels, and bioenergetic status) that occur during doxorubicin challenge. Therefore, at harvest, all cells from the same strain and culture group were pooled, and samples taken for the individual biochemical assays. Further, for some of the experiments, cell strains were concurrently plated in six-well plates (initial viability studies), chamber slides (assessment of DNA

synthesis or induction of apoptosis), or in petri dishes (electron microscopy) to permit ongoing assessment of these parameters.

Isolation, Culture, and Doxorubicin Challenge of Cells

All cellular strains used in this study had undergone < 20 population doubling levels. At harvest, cell counts (standard hemocytometer) and cellular viability (exclusion of trypan blue) were obtained for all cultures. Because doxorubicin exposure did result in some loss of cellular adherence, during the doxorubicin experiments both the medium (which contained floating cells) and the harvested cells were centrifuged for culture viability assessment. In selected experiments, viability assessment by trypan blue exclusion was compared to lactate dehydrogenase release. These two viability assays showed excellent agreement, with viabilities determined by both methods comparing within 1%.

Doxorubicin was added to the medium on the day of assay. Further, because of the propensity for doxorubicin to adhere to methylcellulose filters [Bosanquet, 1986], the concentration of doxorubicin in the medium delivered to the cells was determined by HPLC following sterile filtration. HPLC analyses were conducted on a Beckman Ultrasphere column using a 20 mM acetonitrile potassium phosphate buffer (75:25, pH 4.0) at a flow rate of 0.5 ml/min. Detection was at 254 nm, and comparisons were made against a six point standard curve. No evidence of extraneous peaks or degradation products was noted in the doxorubicin challenge medium samples.

AIDS-KS Cells

HIV^+ individuals that had clinical lesions suggestive of KS were referred for participation in this study by the Ohio State University Department of Infectious Disease. Prior to biopsy of the suspected KS lesion, an examination was conducted to determine the extent and clinical presentation of the individuals' lesion(s). A portion of each biopsy was submitted for light microscopic examination to confirm the diagnosis of AIDS-KS.

The AIDS-KS cells were isolated from the tissue specimen as previously described. [Mallery et al., 1994, 1995, 1998]. Prior to the doxorubicin challenge assays, the AIDS-KS cells were cultured in "COMPLETE" medium, which consisted of: M-199 (GIBCO, Grand Island, NY), supplemented with 15 mM HEPES, 0.23 mg/ml l-glutamine, 11 µg/ml Na pyruvate, sodium heparin (Sigma, St. Louis, MO; 90 µg/ml) endothelial cell growth supplement (prepared in-house, from bovine brain, 150 µg/ml), 15% heat inactivated fetal bovine serum (Hyclone, Logan, UT), 5% heat inactivated pooled, male human serum (Sigma).

Nonlesional Fibroblasts From AIDS-KS Donors

At either the initial biopsy appointment, or at the 1-week recall examination, nonlesional tissue was obtained from the AIDS-KS donors' clinically normal oral mucosa. A portion of the specimen was submitted for microscopic confirmation of "no pathologic change." Nonlesional fibroblasts were isolated and cultured in the same fashion as the AIDS-KS cells. Nonlesional cells from the AIDS-KS donors were included as the endogenous control in this study.

Normal Fibroblasts From HIV^- Gender and Age Matched Donors

Tissue explants were obtained from normal oral mucosa of HIV-negative men, and a portion of the tissue submitted for light microscopy to confirm a diagnosis of "no pathologic change." Normal fibroblasts were isolated and cultured in the same fashion as the AIDS-KS cells. HIV-negative fibroblasts were included as the overall control cellular population.

For these studies, three AIDS-KS strains (ks.1, ks.2, ks.3), two matched nonlesional strains from the KS donors (ks.1 nonles, ks.2 nonles), and three HIV-negative aged matched male fibroblasts (norm.1, norm.2, norm.3) were used.

Assessment of Ongoing DNA Synthesis by Incorporation of the Thymidine Analogue, Bromodeoxyuridine (BrdU)

Cellular BrdU incorporation was determined in accordance with the methods of Bour et al. and Weidner et al. [Bour et al., 1996; Weidner et al., 1993] Briefly, cultured cells were plated (1.5×10^5 cells total/2 ml final volume) on human fibronectin coated one-well plastic chamber slides (Nalgene Nunc, Fisher Scientific, Pittsburgh, PA), and provided 100 µM bromodeoxyuridine (BrdU) during the following culture conditions: log growth (control), and during challenge with either 7 µM doxorubicin or 25 µM doxorubicin. For studies to assess re-entrance

into the cell cycle following doxorubicin exposure, cells were treated with either 7 μM or 25 μM doxorubicin for 96 h, followed by a 48-h recovery in doxorubicin-free COMPLETE medium containing 100 μM BrdU. After fixation, cells were rinsed twice with PBS, then incubated with DAKO monoclonal mouse anti-BrdU using the LSAB kit (DAKO Corporation, Carpinteria, CA). The color of anti-BrdU antigen was developed by using a substrate-chromagen solution reaction kit (BioGenex, San Ramon, CA), in accordance with the manufacturer's instructions. Finally, the cells were rinsed twice with double distilled water, followed by hematoxylin counterstaining. Positive cells demonstrated an intense brown staining which was restricted to the cellular nucleus.

Quantitation of cellular BrdU incorporation was conducted by counting four random cellular fields of 100 cells in each field. Results are expressed as the % of the total number of cells that incorporated BrdU.

Assessment of Cellular Entrance Into Apoptosis

Two assays (DNA fragmentation and an in situ staining) were used to determine whether or not doxorubicin challenge (at both the 7 μM and 25 μM doses) resulted in the induction of apoptosis. Three cell strains (KS lesional and matched nonlesional, and HIV^- donor) were employed for both assays using both the 7 μM and 25 μM doxorubicin doses.

DNA Fragmentation Assay

The DNA fragmentation assay was conducted in accordance with the method of Swapan et al. [1994] The cells were first challenged with doxorubicin for 4, 12, 24, 48, and 72 h. Control cells from the treated strains were seated at the same densities in doxorubicin-free COMPLETE medium, and then harvested concurrently with the treated cells. Following DNA extraction, the concentration and purity of the DNA was determined spectrophotometrically. The DNA samples (~ 0.9 μg) were run on a 1.8% agarose gel followed by staining with ethidium bromide. This assay employs a biochemical characteristic of apoptosis, i.e., DNA fragmentation restricted to internucleosomal sites, resulting in DNA fragments of multiples of 180–200 bp in size. Therefore, agarose gel electrophoresis displays apoptotic induced DNA fragments in a sequential "ladder" fashion, whereas the DNA cleavage from necrotic cells is random, and consequently appears as a smear.

In Situ Assessment of Apoptosis

The in situ apoptosis induction staining assay was conducted in accordance with the manufacturer's instructions (Apop Tag Plus in Situ Apoptosis Detection Kit, Oncor, Gaithersburg, MD). Control and doxorbucin challenged cells were plated in COMPLETE medium at a density of 50,000 per slide in human fibronectin coated Lab Tek chamber slides, doxorubicin added to the treated cultures, cells harvested after 4, 12, 24, 48, and 72 h, and fixed with 10% ice cold formalin. Positive control cells (obtained from rat mammary glands at the fourth post weaning day) were included with the Oncor staining kit. The negative control consisted of substitution of dH_2O for TdT during the staining procedure. Positive induction of apoptosis was manifested by an intense brown stain restricted to the cellular nucleus, whereas negative results provided a turquoise nuclear stain.

Electron Microscopy

Cellular preparation for electron microscopy was conducted in accordance with the methods of Burry and Lasher and Brinkley et al. [Brinkley et al., 1976; Burry and Lasher, 1978]. Cultured cells were grown on Corning 35 mm plastic petri dishes and either challenged with 25 μM doxorubicin for specified periods of time (24, 48, 72 h; treated cultures) or maintained in COMPLETE medium (control cultures). After fixation, the samples were then dehydrated by processing through ethanol hydroxypropymethacrylate, and then embedded in Epon 812. The sections were cut to 500–800 Å and then stained with lead citrate.

Modulation of Cellular GSH Status Prior to Doxorubicin Challenge

To address the contribution of the cellular thiol redox status in doxorubicin mediated cytotoxicity, cellular GSH status was modified prior to doxorubicin challenge.

GSH Depletion

Cellular levels of GSH were decreased by a method previously employed [Mallery et al., 1991]. Cellular synthesis of GSH was inhibited by a 2-h incubation in "BASE" sera deficient medium containing 1×10^{-4} M buthionine sulf-

oximine, followed by a rinse with room temperature PBS. To decrease the existing levels of GSH, the cultures were then incubated for 1 h in "BASE" medium containing 100 μM 2-cyclohexene-1-one (2 CHX-1-one). We have previously shown that this method is highly effective at reducing cellular GSH levels without promoting intracellular retention of GSSG, and is readily reversible [Mallery et al., 1991]. The cultures were then rinsed with room temperature PBS, and COMPLETE medium either with (treated) or without (control) doxorubicin was added, and the cultures incubated at 37°C, 5% CO_2 until harvest.

Augmentation of Cellular Thiol Status

The "COMPLETE" medium was supplemented with the membrane permeable GSH precursor, 30 mM NAC, for 24 h prior to the introduction of doxorubicin. NAC remained in the medium for the duration of the studies. Control cultures also received 30 mM NAC.

Determination of Cellular GSH Levels

Cellular levels of GSH and the disulfide, GSSG, were determined according to the method of Eyer and Podradsky, 1986 [Eyer and Podradsky, 1986]. NADPH, GSH glutathione reductase (type IV), N-ethylmalemide. and DTNB were obtained from Sigma.

Rapid reaction kinetics were followed on a SLM-AMINCO, Urbana, IL, dual wavelength spectrophotometer, with the following instrument settings: 412 nm vs. 550 nm, 3 nm bandwidth, 0.05 Abs., 50 sec/in. Sample concentration (nmol/ml) were determined by comparison with a 10 point (0.2 to 2 nmol/ml) standard curve conducted concurrently. The results are expressed as nmol GSH or GSSG per mg protein.

Determination of Cellular Bioenergetic Status

Cellular levels of nucleotides and nucleosides were determined by high performance liquid chromatography (HPLC), by a modification of the method of Geisbuhler et al. [1984], as reported in our publications [Mallery et al., 1993, 1994, 1995, 1998]. Nucleotide and nucleoside results are expressed as nmol/mg protein.

Inclusion of Oxidant Scavengers and a Metal Chelator During 25 μM Doxorubicin Challenge

The potential cytoprotective effects of the oxidant scavengers Tempol (Aldrich Chemical Co., Milwaukee, WI) and dimethylthiourea (DTMU; Sigma) and the trace metal chelator desferioxamine (Sigma) were assessed by initiation of a 1-h pretreatment, followed by the scavengers or chelator remaining present during challenge with 25 μM doxorubicin. Control cultures also received the scavengers or chelator, but no doxorubicin.

Protein Determination

Cellular protein levels were determined by the Lowry method, using bovine gamma globulins as the standard protein [Lowry et al., 1951].

Overall Cellular Handling for Biochemical Assessment

In general, cultures were grown to 90% confluency. Where noted, cultures were pretreated with either 30 mM NAC (24 h), 10 mM DTMU or 1 mM desferioxamine (1 h), and GSH depletion (3 h). These pretreatments were followed by 30-min or 24-h incubations with (treated) or without (control) 25 μM doxorubicin. Nineteen culture groups were included in these assays; conditions at harvest were as depicted in Table I. Superscripts denote the numbers assigned to

TABLE I. Nucleotide and GSH Assessment Culture Conditions

Culture condition	Control	Treatment (25 μM dox.)
Log growth	Log control[1]	Log, 30 min dox.[2] Log, 24 h dox.[3]
30 mM NAC	24 h pretreatment[4]	24 h pretx., 30 min dox.[5]
30 mM NAC	48 h treatment[6]	24 h pretx., 24 h dox.[7]
GSH depletion	3 h GSH depletion[8]	GSH depletion, 30 min dox.[9]
GSH depletion	GSH depletion, 24 h rec.[10]	GSH depletion, 24 h dox.[11]
10 mM DMTU	30 min pretx.[12]	30 min. pretx., 30 min dox.[13]
10 mM DMTU	24 h inclusion[14]	pretx., 24 h dox. tx.[15]
1 mM desferioxamine	30 min pretx.[16]	30 min pretx., 30 min dox.[17]
1 mM desferioxamine	24 h inclusion[18]	30 min pretx., 24 h dox.[19]

the specific culture groups. NAC, N acetylcysteine; DMTU, dimethylthiourea.

Statistical Analysis

The ANOVA, followed by the Multiple t-Test was used for the statistical comparisons of the viability and BrdU incorporation data. The two-tailed T-test was employed for the post doxorubicin BrdU recovery assays. Analyses of the treatment course biochemical parameters, e.g., cellular GSH status or ATP levels, were conducted using the Direct Difference Correlated t-Test. Differences of $P \leq 0.05$ were determined to be statistically significant.

RESULTS

Cellular Heterogeneity in Response to 7 μM Doxorubicin

Initial studies were conducted to determine the effects of 7 μM doxorubicin challenge on cellular viability. This initial doxorubicin concentration was selected based on estimates of circulating levels of doxorubicin that are obtained during standard intravenous chemotherapy [Sinha et al., 1987].

These preliminary doxorubicin exposure studies showed both common and diverse cellular responses (Fig. 1A). All five cell strains challenged with doxorubicin showed a decrease in viability over time, with lowest viabilities detected at the final time point (72 h). Further, with the exception of one HIV$^-$ donor normal strain, all control cultures also showed a viability decrease of approximately 5% in the control (doxorubicin-free) cultures during the course of the assay. The first KS strain tested (ks.1) was determined to be statistically significantly more susceptible to doxorubicin mediated cytotoxicity relative to its matched nonlesional cells (ks.1.nonles; Fig. 1B). Notably, at the earliest (4 h) harvest, the ks.1 strain showed an ~25% viability loss relative to its control culture, a finding which likely reflects marked susceptibility of a subpopulation of ks.1 cells to a rapidly occurring doxorubicin initiated plasma membrane perturbation. However, subsequent analyses of other strains (both HIV$^-$ donors and another KS lesional), showed a heterogeneity in cellular response to 7 μM doxorubicin, which was independent of HIV status of donor (Fig. 1A,B). The decreases in cellular viability (% $\bar{\times}$ ± S.D., n = 4 for every group) of the doxorubicin challenged cultures over the experimental time course (harvests at 4, 24, 48, 72 h)

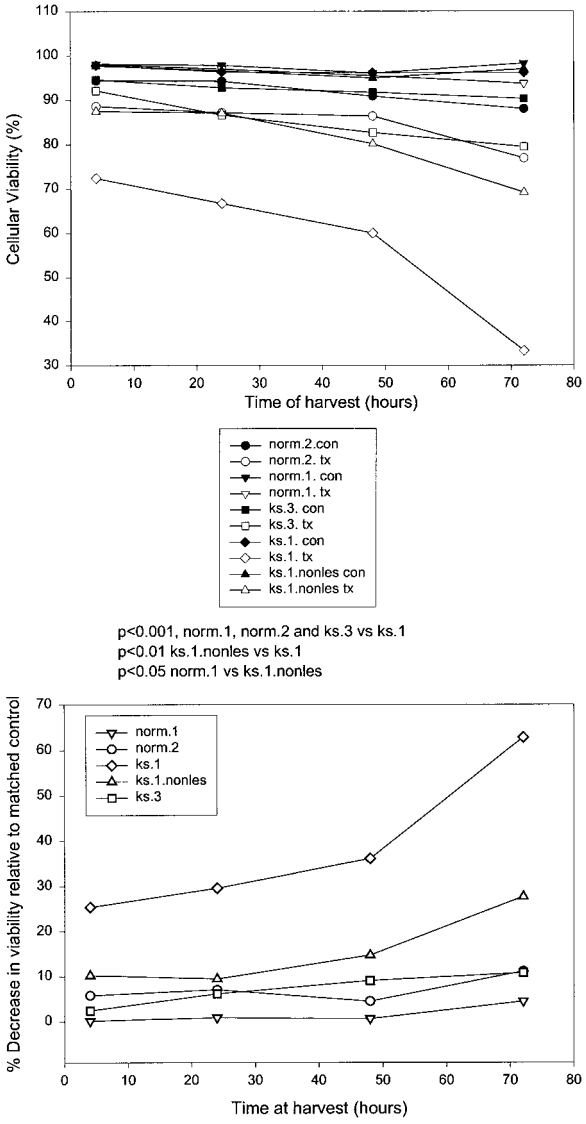

Fig. 1. A,B: Demonstration of the interstrain heterogeneity in response to 7 μM doxorubicin in cultured AIDS-related Kaposi's sarcoma (ks), nonlesional cells from the KS donors (nonles), and HIV$^-$ donor fibroblasts (norm). Log growth cultures were grown in COMPLETE medium containing 7 μM doxorubicin, and harvested at 24, 48, and 72 h for viability assessment via trypan blue exclusion. Statistical analyses to compare the % decrease in viability over time (B) were conducted using the ANOVA followed by the multiple-t-test.

were: ks.1 = 39.9 ± 15.3, ks.1.nonles = 17.7 ± 10.3, ks.3 = 7.8 ± 3.6, norm.1 = 1.6 ± 1.8, norm.2 = 7.8 ± 2.9. Relative to the ks.1 strain, all four additional strains tested were significantly less susceptible to 7 μM doxorubicin's cytotoxic effects [$P < 0.05$ (ks.1.nonles), $P < 0.001$(ks.3, norm.1, norm.2)]. In addition, these significant differences are maintained if the data is normalized to the corresponding control

culture at that time point (Fig. 1B); comparisons which take into account the interstrain differences in control culture viabilities.

Subsequent studies were then conducted to assess the capacity of 7 μM doxorubicin to inhibit DNA synthesis. As can be appreciated from Figure 2A, these studies also demonstrated interstrain differences regarding doxorubicin induced perturbations of DNA synthesis. A common finding among three cell strains was that the control population BrdU indices increased over time in culture, reflective of ongoing cell cycle progression. However, while the control ks.1.nonles cells initially showed the highest BrdU labeling index, their DNA synthesis plateaued by 72 h, a finding which correlated with this culture being postconfluent at harvest. Predictably, doxorubicin inclusion diminished BrdU incorporation in all cellular populations, with the greatest inhibition noted in the strains showing the highest levels of DNA synthesis (Fig. 2B). Additional studies to determine cellular capacity to continue DNA synthesis after a 96-h challenge with 7 μM doxorubicin (a 48-h doxorubicin-free recovery) showed that HIV^- donor cells demonstrated statistically significantly higher BrdU incorporation relative to KS cells ($\overline{\times}$ % BrdU incorporation ± S.D. 55.4 ± 1.67 and 42.4 ± 0.99, n = 3, $P < 0.001$).

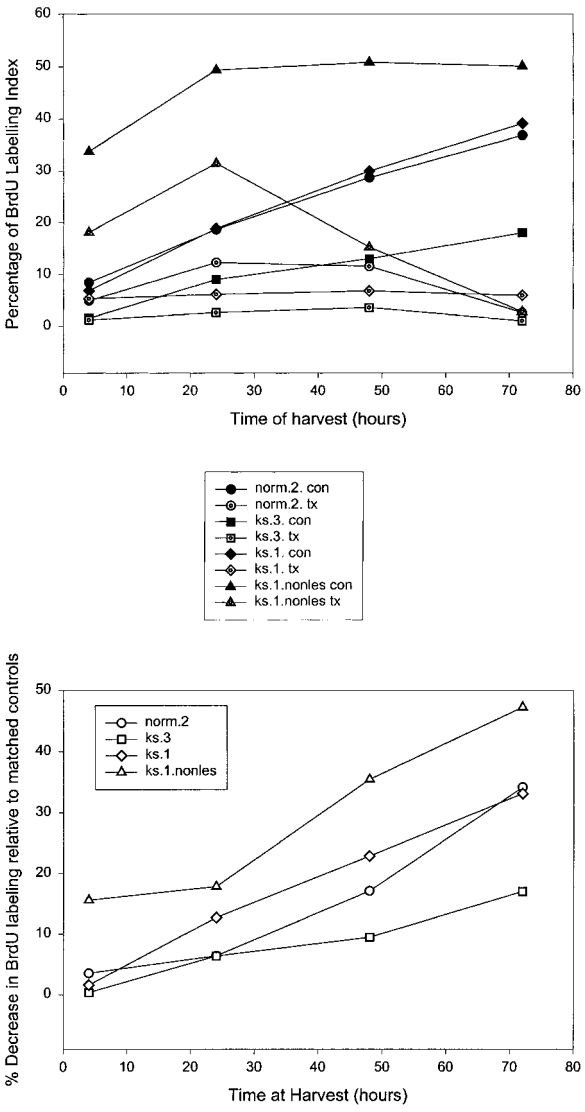

Fig. 2. **A,B**: Comparison of the efficacy of 7 μM doxorubicin to inhibit DNA synthesis in cultured AIDS-KS cells (ks), nonlesional cells from the KS donors (nonles), and HIV^- donor fibroblasts (norm). Log growth cultures were grown in COMPLETE medium containing 100 μM bromodeoxyuridine (BrdU). The treatment cultures concurrently received 7 μM doxorubicin. Cells on tissue culture slides were fixed at 4, 24, 48, 72 h and cells undergoing DNA synthesis determined by cellular incorporation of BrdU. Quantitation of BrdU incorporation was conducted by counting fourrandom cellular fields of 100 cells in each field. Results are expressed as the % of the total number of cells that incorporated BrdU.

While our preliminary studies showed that although doxorubicin diminished DNA synthesis in all cell types, a 7 μM dose was not cytotoxic for all of the KS lesional cells. Therefore, additional studies were conducted using a higher (25 μM) dose of doxorubicin.

Failure of Either 7 μM or 25 μM Doxorubicin to Induce Apoptosis

Neither of the apoptosis assessment assays, conducted at both the 7 μM and 25 μM doxorubicin concentrations, showed induction of apoptosis in any of the strains tested (lesional, nonlesional, and normal). The DNA fragmentation assays revealed smeared DNA segments indicative of random DNA fragmentation. Further, the in situ staining assays did not show any intense brown (positive) nuclear staining in any of the doxorubicin-treated cells (data not shown). Collectively, these results suggest that although doxorubicin challenge did result in some cytotoxicity, cell death proceeded without inducing apoptosis.

HIV^- Donor Fibroblasts Demonstrate Higher Rates of DNA Synthesis After Doxorubicin Challenge

Because one of the putative cytotoxic mechanisms of action of doxorubicin is to disrupt the function of topoisomerase II, studies were conducted to determine cellular capacity to continue and sustain DNA synthesis both during

and following 25 μM doxorubicin exposure. HIV⁻ donor and KS lesional cells were challenged with 25 μM doxorubicin for 96 h, followed by a 48-h recovery in doxorubicin free medium which contained BrdU.

Timed harvests (4, 24, 48, 72, 96 h) during the doxorubicin challenge showed that while doxorubicin decreased DNA synthesis in both groups, KS lesional cellular DNA synthesis was significantly inhibited relative to HIV-negative donor cells (Fig. 3A). Further, while both groups showed comparable 48 h BrdU labeling indices of 94.7% and 94.6% during log growth, the HIV⁻ donor fibroblasts sustained statistically significantly higher BrdU indices at the 48 h recovery ($P \leq 0.001$; Fig. 3B). However, while the KS lesional BrdU indices remained significantly lower, the relative increase in the percentage of cells in S phase following doxorubicin challenge was greater in the KS lesional cells (74.1% vs. 32.8%; Fig. 3B).

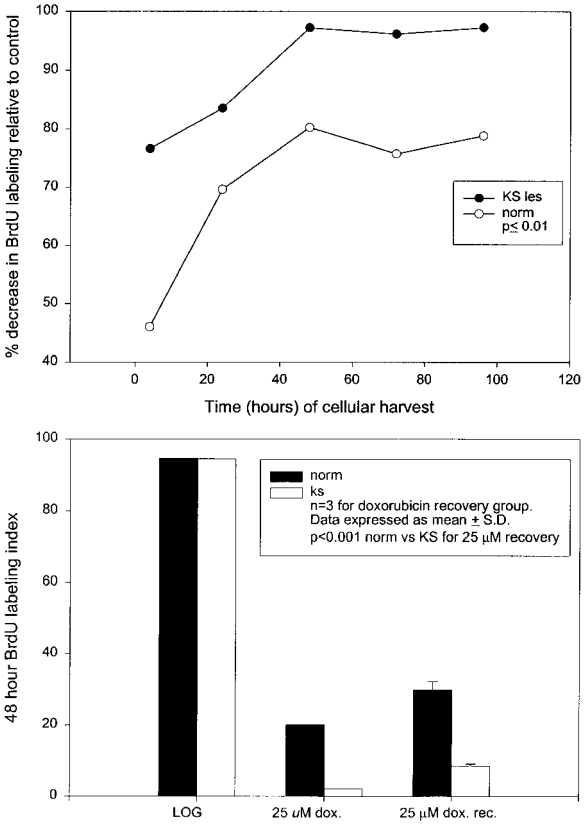

Fig. 3. A,B: Demonstration of enhanced capacity of control (HIV-negative donor) fibroblasts to continue and sustain DNA synthesis during and following 25 μM doxorubicin challenge. AIDS-KS cells and normal control fibroblasts were challenged with 25 μM doxorubicin (with harvests at 4, 24, 48, 72, and 96 h), and then cultured for 48 h in doxorubicin-free COMPLETE medium. Statistical analyses were conducted using the Direct Difference Correlated t Test (A) and the Two Tailed Student's *t* test (B).

Subsequent studies were then conducted to test our hypothesis that the cellular thiol redox status and ROI scavenging capacities modulates cellular susceptibility to doxorubicin. These experiments were designed to provide a time course analysis of redox relevant biochemical parameters (bioenergetic profiles and glutathione [GSH] status) during doxorubicin challenge. A variety of experimental conditions were employed including incorporation of agents which either augmented or reduced cellular capacity to withstand oxidant stress (Table I).

Presence of 25 μM Doxorubicin Affects the Cellular Bioenergetic Profile

The cellular energy charges ([ATP]+ ½ [ADP]/[ATP]+[ADP]+[AMP]) showed three trends during the course of 25 μM doxorubicin challenge (Table II). Early in the course of doxorubicin challenge (30-min harvest), there was little or no change in the cellular energy charge. Secondly, by the 24-h time point, most of the strains demonstrated a decreased energy charge in response to doxorubicin treatment. An exception were the cultures that received NAC during doxorubicin challenge as these cultures maintained their energy charge (e.c. ≥ 0.90 at the 24-h harvest during NAC+doxorubicin) [except in one ks strain (ks.1)]. Finally, while GSH depletion prior to doxorubicin challenge did adversely affect many cellular parameters such as viabilities, GSH depletion did not result in energy charge differences relative to non GSH deleted, doxorubicin challenged cultures.

The Cellular Thiol Redox Status Correlates With the Retention of Cellular Viability During 25 μM Doxorubicin Challenge

Consistent with our previous findings [Mallery et al., 1994, 1995, 1998], GSH levels were determined to be significantly higher in healthy HIV⁻ donor fibroblasts relative to both KS lesional or nonlesional cells (Fig. 4A,B). As previously noted [Mallery et al., 1998], the inclusion of NAC most dramatically augmented KS lesional GSH levels as 1.5-, 4-, and 7-fold increases in GSH levels were detected (relative to log control) in the NAC control cultures for the

TABLE II. Alterations in Cellular Energy Charge ([ATP] + ½[ADP]/[ATP] + [ADP] + [AMP]) During 25 μM Doxorubicin Challenge[a]

Cell strain	Log 30 min con[1], tx[2]	log, 24 h con[1], tx[3]	NAC 30 min con[4], tx	NAC 24 h con[6], tx[7]	↓GSH 30 min con[8], tx[9]	↓GSH 24 h con[10], tx[11]
norm.3	0.98 con[1]	0.98 con[1]	0.89 con[4]	0.84 con[6]	0.98 con[8]	0.97 con[10]
	0.97 tx[2]	0.80 tx[3]	0.99 tx[5]	0.91 tx[7]	not cond.[b]	0.78 tx[11]
ks.3	0.96 con[1]	0.96 con[1]	0.95 con[4]	0.95 con[6]	0.96 con[8]	0.95 con[10]
	0.91 tx[2]	0.78 tx[3]	0.93 tx[5]	0.92 tx[7]	0.97 tx[9]	0.78 tx[11]
ks.1	0.95 con[1]	0.95 con[1]	not. cond.[b]	0.92 con[6]	0.75 con[8]	0.77 con[10]
	0.81 tx[2]	0.85 tx[3]	0.72 tx[5]	0.58 tx[7]	0.84 tx[9]	0.64 tx[11]
ks.1.nonles	0.91 con[1]	0.91 con[1]	0.89 con[4]	0.92 con[6]	0.93 con[8]	0.88 con[10]
	0.95 tx[2]	0.71 tx[3]	0.93 tx[5]	0.92 tx[7]	0.90 tx[9]	0.80 tx[11]
norm.2	0.93 con[1]	0.93 con[1]	0.94 con[4]	0.59 con[6]	0.91 con[8]	0.54 con[10]
	0.97 tx[2]	0.62 tx[3]	0.86 tx[5]	0.90 tx[7]	0.91 tx[9]	0.63 tx[11]

[a]Superscripts correspond to experimental groups as described in Table I.
[b]not cond., not conducted.

normal, nonlesional and KS lesional cultures, respectively (data not shown). However, even during NAC supplementation, GSH levels were still appreciably lower in the KS cultures. Notably, the uniformly low GSH levels detected in selected treatment groups reflect experimental conditions, i.e., GSH depletion without recovery (control, #8), and GSH depletion immediately followed by a 30-min doxorubicin challenge (treatment, #9). Further, the oxidized form of GSH, GSSG, was exclusively detected in the KS strains (ks.1 and ks.3) only during the 30 min doxorubicin challenge of log growth cultures. These two KS strains contained 2% (ks.1) and 3% (ks.3) of total GSH present as GSSG.

Both cellular viability and energy charge decreased over time of doxorubicin challenge, findings which correspond to the energy requiring active transport mechanisms necessary to maintain membrane integrity (Tables II, III). However, although NAC incorporation benefited the cellular energy charge during doxorubicin exposure, NAC had variable effects on cellular viabilities. Also, after GSH depletion, doxorubicin was most cytotoxic, as manifest by the greatest decreases in viabilities in the GSH depletion, 24-h doxorubicin treatment cultures (group 11).

Notably, during the most stringent conditions (GSH depletion followed by 24-h doxorubicin challenge, italicized data in Table III), there is a strong correlation ($r = 0.94$) between cellular GSH status and cellular viability, i.e., higher GSH levels corresponded to higher viabilities, with the highest GSH levels and viabilities present in the normal cultures (Fig. 5A).

Cellular NADPH Levels Also Correlate With Both Retention of Viability and GSH Status During Doxorubicin Challenge

Consistent with our previous findings [Mallery et al., 1994, 1995, 1998], log growth NADPH levels were determined to be higher in HIV^- donor control cells relative to KS lesional or nonlesional cultures (Table IV). Further, total nicotinamide nucleotide levels during log growth showed that the HIV^- donor control cultures possessed increased nicotinamide nucleotides ([NAD(H)] and [NADP(H)]) relative to KS lesional or nonlesional cultures. Relative to the KS cultures, the HIV^- donor cultures contained 1.6-fold higher levels of NAD(H) [13.9 vs. 8.4 (nmol/mg)] and 2-fold higher levels of NADP(H) 2-fold [0.8 vs. 0.4 (nmol/mg)]. Comparable differences were found in comparison of the normal controls relative to the nonlesional cultures. Also, while total phosphorylated adenine nucleotide levels (ATP + ADP + AMP) during log growth were similar (normal control = 28, KS = 23), the normal control cultures contained 1.6-fold greater ATP/ADP ratios (23 vs. 14).

The data depicted in Figure 5B show a strong correlation ($r = 0.93$) between cellular NADPH levels and retention of cellular viability during doxorubicin's most cytotoxic conditions (GSH depletion, followed by 24 h 25 μM doxorubicin challenge). Similar to the GSH-viability correlation, the highest NADPH levels and cellular viabilities were found in the normal cultures. Not unexpectedly, there is also a strong positive

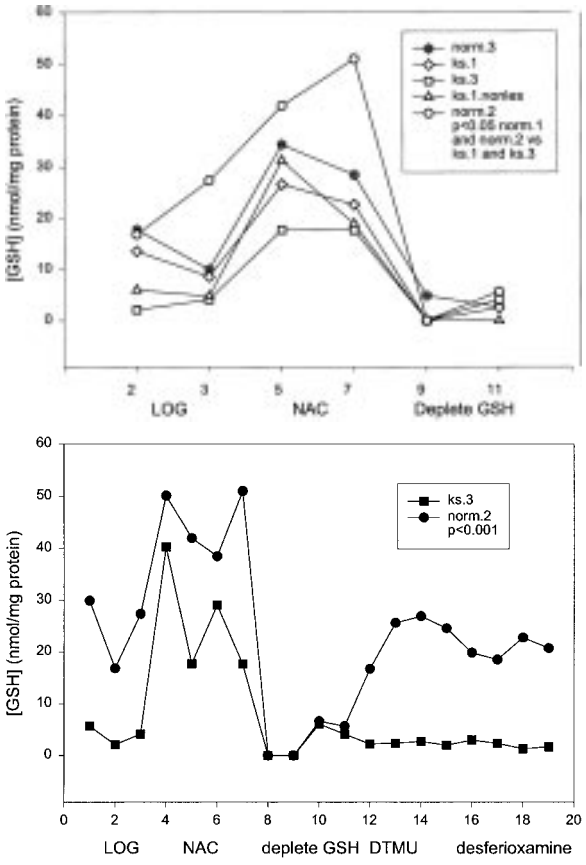

Fig. 4. **A**: Enhanced capacity of control (HIV⁻ donor) fibroblasts to upregulate GSH levels during 25 μM doxorubicin challenge. Cells were incubated with 25 μM doxorubicin for either 30 min or 24 h during: log growth, after a 24 h pretreatment with 30 mM N-acetylcysteine (NAC), and after a 3 h GSH depletion. Statistical analyses were conducted using the Direct Difference Method for Correlated t test. **B**: Demonstration of higher GSH levels of HIV⁻ donor control fibroblasts during all culture conditions, regardless of inclusion or exclusion of doxorubicin. Cultures were grown to 90% confluency. Where noted, cultures were pretreated with either 30 mM NAC (24 h), 10 mM dimethylthiourea (DTMU) or 1 mM desferioxamine (1 h), and GSH depletion (3 h). These pretreatments were followed by 30 min or 24 h incubations with (tx) or without (con) 25 μM doxorubicin. For every group the numbering is as follows: 30 min control, 30 min treat; 24 h control, 24 h treat. Statistical analyses were conducted using the Direct Difference Method for Correlated t test.

correlation (r = 0.93) between cellular GSH and NADPH levels (Fig. 5C); findings which are consistent with the reliance of GSSG reductase on NADPH for the provision of reducing equivalents.

Normal Fibroblasts Retain Greater Cell Viability During a 96 h Time Course Doxorubicin Challenge

Two of the same strains which were employed for the biochemical assays (ks.3, norm.3) were also used for a concurrent 96 h time course viability assessment during 25 μM doxorubicin challenge. These studies show that relative to the KS lesional cells, normal fibroblasts retained statistically significantly higher viabilities over the 96 h course of doxorubicin exposure (Fig. 5D).

During a 72 h Challenge With Doxorubicin, Nucleotide Profiles of Normal, and Nonlesional Cells Show Enhanced Tolerance Relative to KS Lesional Cells

Because the 24-h doxorubicin studies suggested cumulative damage to the cellular energy status over time, additional 72-h time course analyses were conducted on KS lesional (ks.2), matched nonlesional (ks.2.nonles), and HIV⁻ levels (norm.2). We speculated that this longer duration would permit identification of strains most susceptible to doxorubicin mediated bioenergetic perturbations. As in the shorter duration studies, these 72-h assays also demonstrated that AIDS-KS cells possessed a reduced capacity to tolerate doxorubicin challenge (Table V). These data show that relative to matched nonlesional or control normal cells, AIDS-KS cells possess appreciably lower levels of ATP, NADPH and a diminished energy charge following 72 h of doxorubicin exposure. This KS nucleotide profile, in conjunction with the marked concurrent increase in the KS cells' redox state imply an ongoing reductive stress which may reflect mitochondrial failure.

While the inclusion of doxorubicin was associated with a reduction in cellular viability, cell numbers at harvest were actually higher in the treatment relative to the comparable control cultures.

Qualitative Analyses Also Support That AIDS-KS Cells are More Susceptible to Doxorubicin, and Suggest That Mitochondria are Targeted

Following 24 h of 25 μM doxorubicin challenge, all strains showed some evidence of ongoing stress including reduction in adherence and cytoskeletal alterations. However, these changes were manifest at earlier times and to a greater extent in the AIDS-KS cultures (Fig. 6). Further, while GSH depletion per se did not result in marked visible perturbations in these strains, the combination of GSH depletion and doxorubicin challenge provided qualitative findings

TABLE III. Effect of Modulation of GSH Status on Cellular Viability During 25 μM Doxorubicin Challenge[a]

Cell strain	log 30 min tx^{2}	log 24 h tx^{3}	NAC 30 min tx^{5}	NAC 24 h tx^{7}	↓GSH 30 min tx^{9}	↓GSH 24 h tx^{11}	[GSH] 24 h tx^{11} (nmol/mg)
norm.3	−1.45	−5.09	+3.94	−1.97	−4.97	−8.69	4.85
ks.3	−2.18	−6.38	−0.1	−2.23	−1.61	−19.00	4.12
ks.1	−3.93	−10.57	+2.11	−18.70	−7.27	−30.71	2.50
ks.1.nonles	−6.82	−18.28	−10.06	−32.81	−21.04	−37.49	0.00
norm.2	−1.52	−1.98	0.00	−2.60	−8.57	−9.79	5.67

[a]Data is expressed as the percent reduction in culture viability relative to the corresponding log growth control culture.

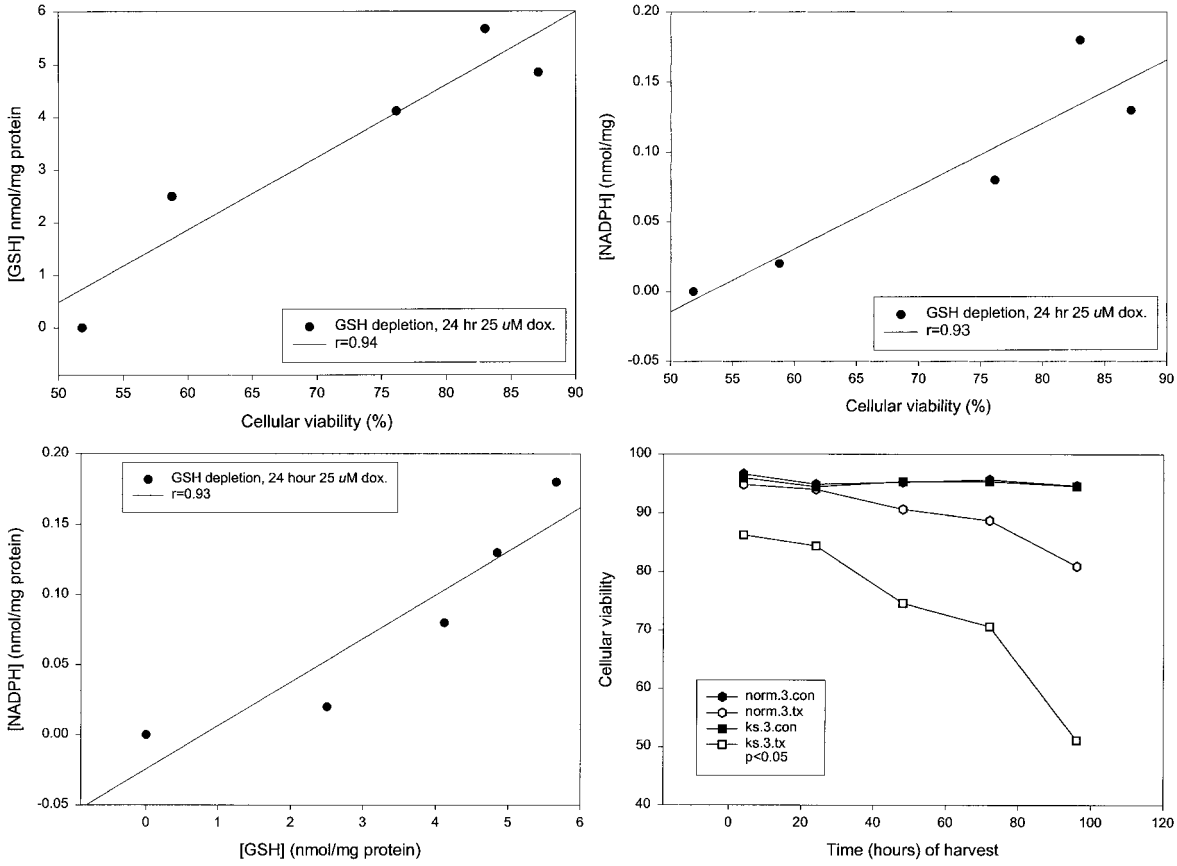

Fig. 5. Demonstration of the positive correlations between cellular GSH levels and viability **(A)**, levels of NADPH and viability **(B)**, and cellular GSH and NADPH levels **(C)**. Cells were grown to 90% confluency in COMPLETE medium, pretreated for 3 h to deplete cellular GSH levels, followed by a 24 h challenge with 25 μM doxorubicin. Cellular viabilities, GSH, and NADPH levels were determined by trypan blue exclusion, dual beam spectrophotometric assay, HPLC, respectively. **D**: Enhanced capacities of HIV⁻ donor fibroblasts to retain viability during a 96 h time course challenge with 25 μM doxorubicin. Cells were grown to 90% confluency in COMPLETE medium, then challenged with 25 μM doxorubicin and harvested at 4, 24, 48, 72, and 96 h. Cellular viability was determined by trypan blue exclusion. Statistical analyses were conducted using the Direct Difference Method for Correlated t Test.

which were consistent with the nucleotide and viability data (Fig. 6).

The nucleotide analyses suggested that mitochondria were a target for doxorubicin's cytotoxic effects. Qualitative cellular assessments at the ultrastructural level supported this premise (Fig. 7) Doxorubicin challenge was accompanied by perturbations which included mitochondrial swelling, reduction in mitochondrial number, and loss of definition in mitochondrial cristae and mitochondrial outer membranes. Ultrastructural changes were also

TABLE IV. Effect of 25 μM Doxorubicin Challenge on Cellular NADPH Levels[a]

Cell strain	Log control[1]	Log 30 min tx[2]	Log 24 h tx[3]	NAC 30 min con[4] tx[5]	NAC 24 h con[6] tx[7]	↓GSH, 30 min con[8] tx[9]	↓GSH, 24 h con[10] tx[11]
norm.3	0.51	0.56	0.18	0.21^{1con}	0.05^{3con}	0.11^{5con}	0.38^{7con}
				0.28^{2tx}	0.11^{4tx}	not cond[6]	0.13^{8tx}
ks.3	0.14	0.39	0.00	0.32^{1con}	0.30^{3con}	0.26^{5con}	0.23^{7con}
				0.13^{2tx}	0.36^{4tx}	0.08^{6tx}	0.08^{8tx}
ks.1	0.09	0.05	0.07	not cond	0.00^{3con}	0.00^{5con}	0.00^{7con}
				0.00^{2tx}	0.00^{4tx}	0.00^{6tx}	0.02^{8tx}
ks.1.nonles	0.12	0.00	0.11	0.47^{1con}	0.09^{3con}	0.00^{5con}	0.19^{7con}
				0.26^{2tx}	0.15^{4tx}	0.00^{6tx}	0.00^{8tx}
norm.2	0.19	0.19	0.14	0.27^{1con}	0.15^{3con}	0.54^{5con}	0.32^{7con}
				0.20^{2tx}	0.20^{4tx}	0.32^{6tx}	0.18^{8tx}

[a]Data expressed as nmol/mg protein.

TABLE V. Effect of 72 h Incubation With 25 μM Doxorubicin on Cellular Bioenergetic Status and Viability[a]

Cell strain	[ATP]	[NADPH]	Energy charge	Redox state	↓Viability	Cell nos. × 10^6
norm.2.con	28.72	0.53	0.98	0.71		3.66
norm.2.tx	15.65	0.68	0.71	0.71	29.4%	4.34
ks.2.nonles.con	35.92	0.13	0.94	0.50		3.65
ks.2.nonles.tx	13.47	0.12	0.82	0.60	29.4%	4.37
ks.2..con	28.12	0.43	0.97	0.79		2.13
ks.2.tx	3.20	0.00	0.47	1.54	36.4%	3.72

[a]ATP and NADPH expressed as nmol/mg protein, energy charge = ATP + 1/2 ADP/ATP + ADP + AMP, redox state = NAD(P)H/NAD(P)$^+$, ↓viability = % decrease in viability relative to control, con = log growth, tx = 72 h incubation with 25 μM doxorubicin.

apparent at other cellular sites including the plasma membrane (bleb formation, overall thickening and loss of distinction), and intracytoplasmic vacuole formation.

Inclusion of DMTU and Desferioxamine Benefits KS Cells, But is Ineffective in Normal Controls

A final series of experiments were conducted to determine whether or not agents which diminished the potential for Fenton chemistry affected cellular response to doxorubicin. The results of these assays, show that AIDS-KS cells (ks.3) benefited from inclusion of either DMTU or desferioxamine, whereas these agents were largely ineffective, or deleterious to normal control cells (norm.2; Fig. 8A,B). While the viabilities of normal control cells decreased during the inclusion of either DMTU or desferioxamine, the KS cellular viabilities were significantly increased in the presence of these agents (Fig. 8A). Decreases in normal cellular viability were noted in both the control and treatment groups, although the decreases were most dramatic in the desferioxamine 25 μM doxorubicin treatment cultures. Further, these results were not restricted to these cellular strains. While a second KS strain tested (ks.1) also showed similar beneficial responses to hydroxyl radical scavenger and iron chelator inclusion (approximate 10% increase in cellular viability relative to matched log treatment groups), these agents were deleterious to a second normal culture (norm.3).

In addition, while the inclusion of DMTU and desferioxamine decreased the ATP levels of the normal control cells relative to comparable log treated cultures, the converse effect was noted in the KS cells (Fig. 8B).

DISCUSSION

The present treatments for AIDS-KS include surgery, radiation, and systemic chemotherapy, all of which are associated with significant morbidity [Coukell and Spencer, 1997; Northfelt, 1994; Tappero et al., 1993]. Further, although doxorubicin is one of the more commonly employed and effective of the AIDS-KS chemotherapeutic agents, its cytotoxic mechanism

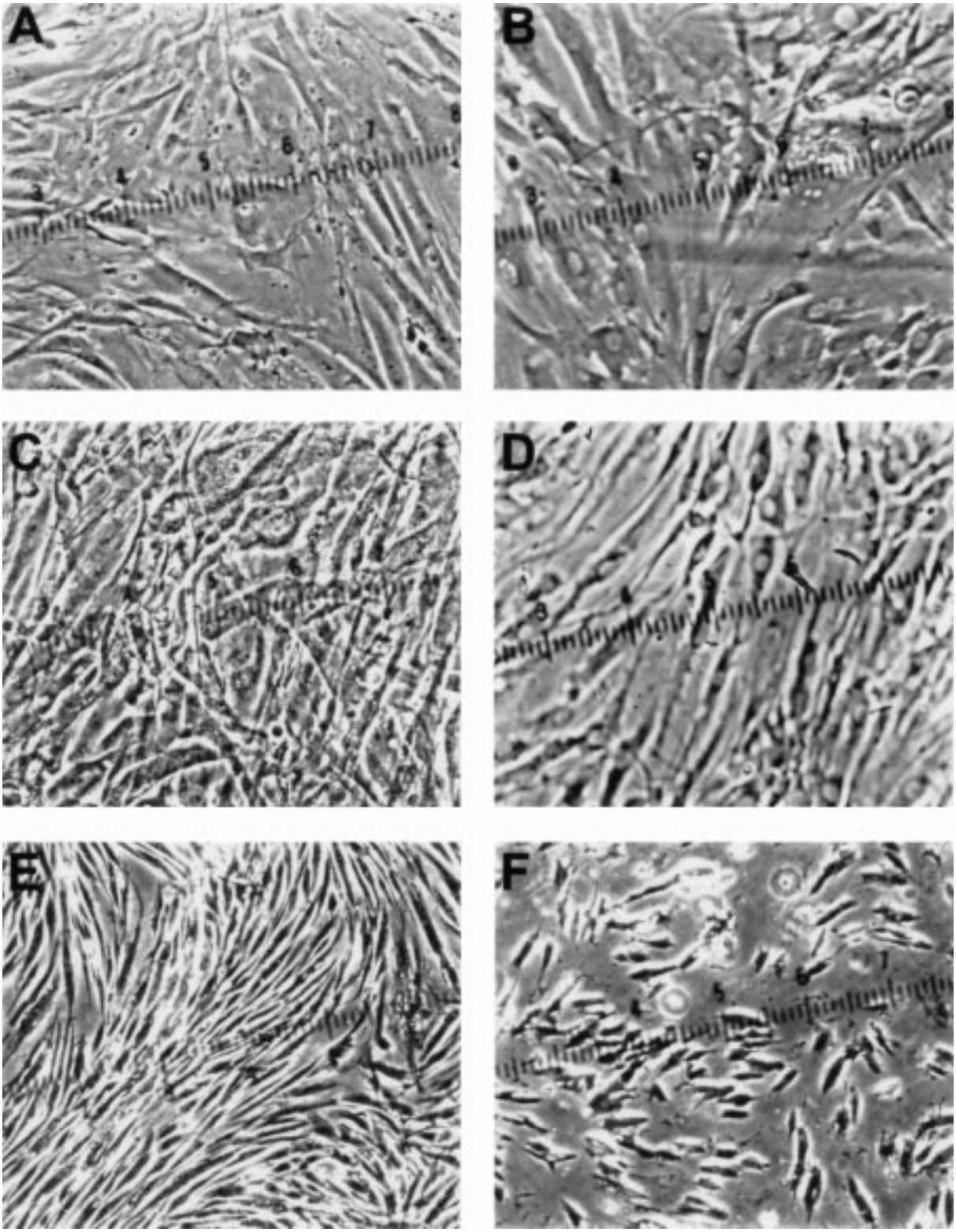

Fig. 6. Qualitative cellular differences were apparent between AIDS-KS and HIV$^-$ donor cultures during both log growth as well as doxorubicin challenged conditions. During log growth control fibroblasts [norm.3**(A)**] cultures demonstrated a uniform cellular appearance and monolayer growth, while AIDS-KS cells [ks.3**(C)**] showed both a greater degree of cellular pleomorphism and loss of contact inhibition. Further, although all culture groups showed alterations during 25 μM doxorubicin challenge, cytoskeletal perturbations and loss of plasma membrane and nucleolar distinctions were more apparent in the KS cultures [norm.3**(B)**], [ks.3**(D)**]. The most dramatic cellular perturbations were noted in the KS cultures which were doxorubicin challenged after GSH depletion. While GSH depletion protocol alone resulted in an elongated, spindled phenotype [ks.3, 24 h after GSH depletion**(E)**], GSH depletion followed by a 24 h 25 μM doxorubicin challenge [ks.3**(F)**] caused overt cell lysis and markedly mishapen cells. Photomicrographs: × 10 image scale E, F; × 25 image scale A–D.

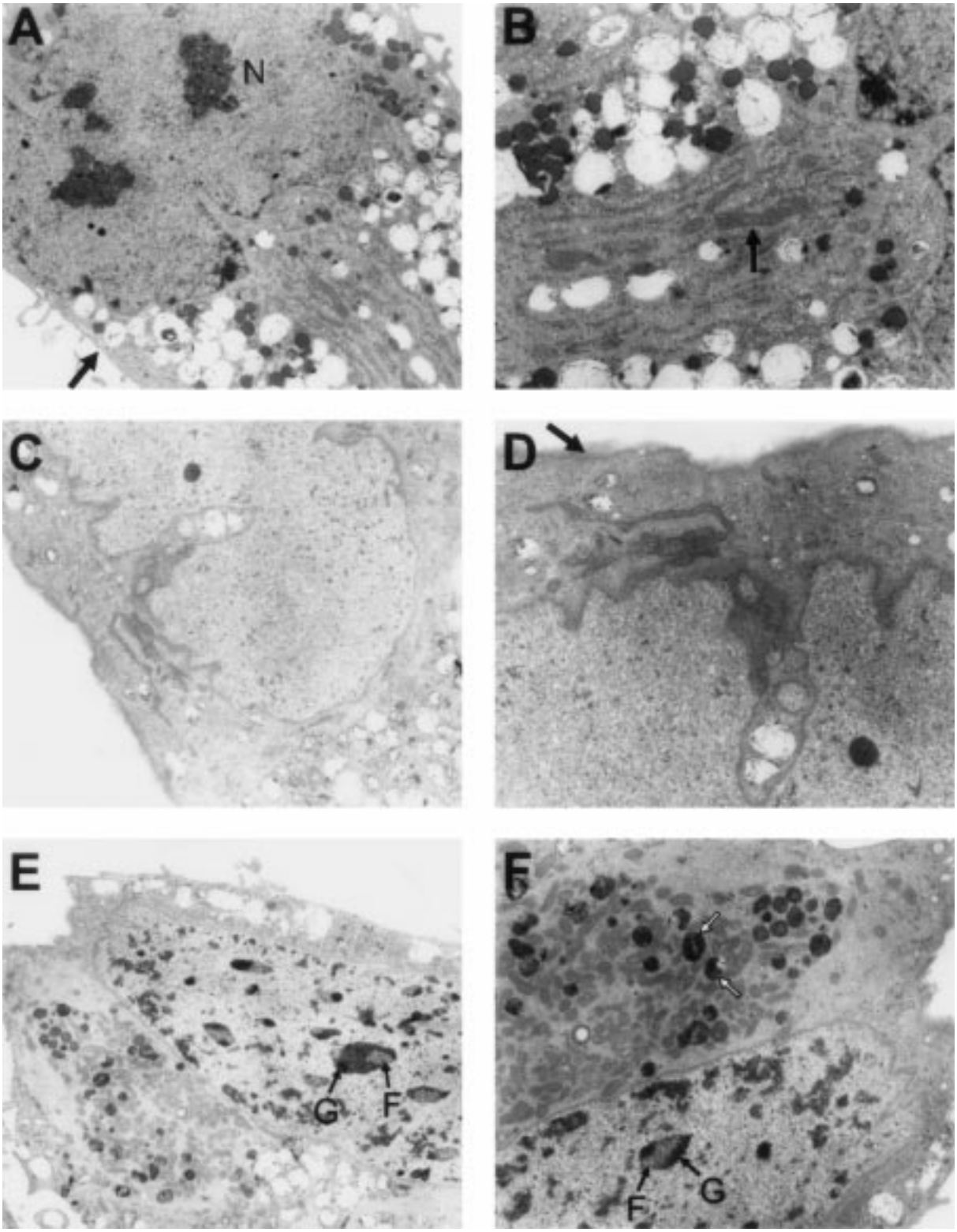

Fig. 7. AIDS-KS cellular ultrastructural appearances correlated with their corresponding bioenergetic profiles. During proliferative growth [ks.3 **(A)**, **(B)**] the plasma membrane (arrow, A) and mitochondrial membranes and cristae (arrow, B) were distinct and well delineated. Also, the granular and fibrillar components remain randomly arranged in a normal nucleolus (N). However, after a 24 h challenge with 25 μM doxorubicin, plasma membrane perturbations were apparent including bleb formation and loss of delineation (arrow, D) [ks.3**(C),(D)**]. GSH depletion followed by a 24 h 25 μM doxorubicin challenge [ks.3 **(E), (F)**] caused mitochondrial swelling and reduction in cristae as well as formation of electron dense bodies resembling degenerating mitochondria (arrows, F). In addition, GSH depletion and doxorubicin challenge was accompanied by contraction of the nucleolus and a distinct segregation of its granular **(G)** and fibrillar (F) components (E, F). Magnifications: × 12,500 A, C, E; × 20,000 B, D, F.

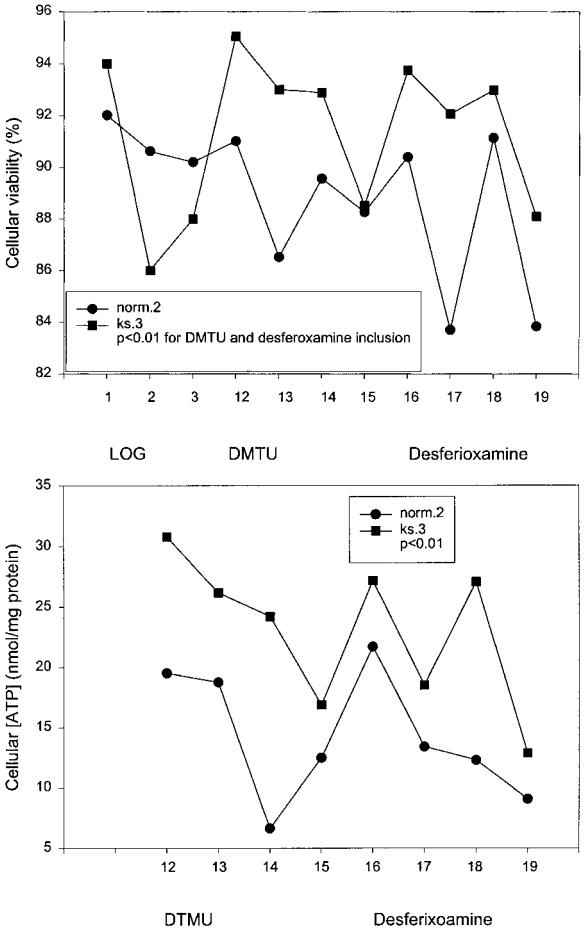

Fig. 8. **A,B**: Comparison of the effects of a $OH^{\cdot}$ radical scavenger and an iron chelator on cellular viabilities and ATP levels during doxorubicin challenge. AIDS-KS cells and HIV^{-} donor fibroblasts were cultured in COMPLETE medium, and where noted, were pretreated with 10 mM dimethylthiourea (DMTU) or 1 mM desferioxamine 30 min prior to challenge with 25 μM doxorubicin. For every group given either DMTU or desferioxamine the data is depicted as follows: 30 min control, 30 min treat; 24 h control, 24 h treat. The log cultures consisted of: proliferative growth control, 30 min treat and 24 h treat. The numbers on the X axis correspond to the cellular groups as described in detail in Table I. While the viabilities of control cells decreased after addition of either DMTU or desferioxamine, the KS cellular viabilities were significantly increased in the presence of these agents (A, $P < 0.01$). Also, while the inclusion of DMTU or desferioxamine decreased HIV^{-} donor cellular ATP levels relative to comparable log treated cultures, the converse effect was seen in the KS cells (B, $P < 0.01$). Statistical analyses were conducted using the Direct Difference Method for Correlated t test.

against AIDS-KS cells has not previously been determined. Our results show that generation of ROI is attributable, at least partially, for doxorubicin's cytotoxic effects on AIDS-KS cells. Our data also demonstrate that doxorubicin tolerance is correlated with cellular ability to maintain NADPH and GSH levels, and that AIDS-KS are more susceptible to doxorubicin as a consequence of an impaired capacity to maintain these thiol redox pools. Insight into the mechanism(s) by which agents like doxorubicin mediate cytotoxicity may direct future research toward drug formulations which could capitalize on the inherent AIDS-KS cellular susceptibility to oxidant stress. e.g.. a more redox active doxorubicin derivative.

The interstrain heterogeneous responses observed during 7 μM doxorubicin challenge were not unexpected, and in agreement with findings regarding doxorubicin toxicity from other laboratories [Lee et al., 1989; Mestdagh et al., 1994; Sinha et al., 1987]. Because all the cellular populations used in our studies were derived from the outbred human population, it was anticipated that there would be individual differences in drug inactivation and metabolizing capacities [Willey et al., 1996]. Two parameters, i.e., retention of viability and continuation of DNA synthesis were assessed in these preliminary studies. The viability data revealed one highly doxorubicin susceptible cell strain (ks.1), and similar to our previous findings [Mallery et al., 1998] the nonlesional cells from this donor did show an "intermediate" phenotype, with viabilities interspersed between the ks.1 lesional and other control and ks strains. Another KS strain (ks.3) showed very low control and treatment BrdU labeling indices, suggesting that this strain may be manifesting cell cycle perturbations common to some neoplastic cells, e.g., mitotic lag [Pitot, 1986]. Further, although these preliminary studies showed AIDS-KS interstrain heterogeneous responses to 7 μM doxorubicin, following challenge with the higher, potentially more redox active doxorubicin dose, the KS cells from different donors uniformly demonstrated enhanced doxorubicin mediated toxicity relative to HIV-negative donor control cells.

There are several potential explanations why doxorubicin failed to induce apoptosis in our cell populations. Although apoptosis may be initiated by a variety of environmental stresses such as oxidants, ionizing radiation or reduction of growth factors, this pathway is ultimately regulated by the ratio of expression of apoptotic promoting and preventing genes such as bax and the bcl family [Bissonnette et al., 1992; Bojes et al., 1997; Boise et al., 1993; Clarke et al., 1993; Fisher et al., 1993]. Rel-

evant to our findings, enhanced expression of the apoptosis inhibiting bcl-2 is associated with increases in intracellular GSH levels [Bojes et al., 1997]. Further, bcl-xl encodes a protein which resembles a pore forming domain that modulates the cellular thiol redox status by regulating plasma membrane and mitochondrial ion channels and GSH efflux [Bojes et al., 1997]. Our data, which show a maintenance of intracellular GSH levels and preservation of mitochondrial oxidative metabolism in the doxorubicin tolerant HIV^- donor cultures, suggests that bcl gene expression was upregulated in these populations during doxorubicin challenge. Alternatively, the energy requirements necessary to enter apoptosis may have been prohibitive for those KS strains which sustained marked doxorubicin associated damage to energy generating organelles like mitochondria [Clarke et al., 1993].

Our results indicated that HIV-negative donors' cells showed significantly higher rates of DNA synthesis both during and subsequent to challenge with both doxorubicin doses. These findings likely reflect the capacity of normal cells to limit doxorubicin associated damage, retain mitochondrial function, and synthesize new enzyme to overcome the doxorubicin-topoisomerase II inhibition [Mathews and van Holde, 1990].

Our data revealed that AIDS-KS cells contained relatively lower levels of nicotinamide nucleotides during proliferative growth, and that NADPH disparities increased following doxorubicin exposure. These differences did not reflect an in vitro "deprivation artifact" as the culture medium contained the nicotinic acid precursors glutamine, tryptophan, and pyridoxine HCl. Our nicotinamide nucleotide data imply that pathways for synthesizing and/or scavenging pyridine nucleotides are impaired in the AIDS-KS cells. Optimally, during challenge with redox active agents such as doxorubicin (which increases the GSSG/GSH ratio) there is a concurrent stimulation of NADPH generating pathways such as the energy dependent transhydrogenase, NAD(H) kinases, and the hexose monophosphate shunt [Meister, 1994]. However, the failure of AIDS-KS cells to increase NADPH levels during doxorubicin induced increases in GSSG suggests that this coupling did not occur in KS strains. Further, the retention of detectable GSSG in AIDS-KS strains reflects the fact that NADPH is the preferred cofactor for GSSG reductase, as the GSSG reductase NADH pair is only ~1% as effective at physiological pH [Kehrer and Lund, 1994; Lui and Kehrer, 1996].

Collectively, our nucleotide and viability results revealed that those cultures that increased or maintained GSH and NADPH levels subsequent to doxorubicin challenge also maintained their energy charge; implying retention of mitochondrial function. Notably, although the mitochondria are the primary suppliers of reducing equivalents, most of these are generated from NADH linked substrates [Mathews and van Holde, 1990]; making energy dependent pyridine nucleotide interconversion and thiol disfulfide interchange necessary for maintenance of mitochondrial NADPH and GSH pools. Further, because mitochondria are highly susceptible to thiol oxidation, reduced GSH is key for maintenance of thiol dependent ion channels and to preserve the function of the primary peroxide degrading enzyme in the mitochondria, GSH peroxidase [Kehrer and Lund, 1994; Meister, 1994]. Other data, which supported the benefits of reduced thiols, were our findings that NAC inclusion was cytoprotective during doxorubicin challenge in all culture groups. The apparent cytotoxicity of NAC towards some HIV^- donor cells in the absence of doxorubicin likely reflects the high endogenous reduced thiol levels in normal cells. Finally, NAC's abilities to serve as a GSH precursor as well scavenge ROI would explain its beneficial effects during doxorubicin challenge [Meister, 1994].

It has become apparent that the mechanisms of doxorubicin induced cytotoxicity are not as straightforward as initially surmised [DeGraff et al., 1994; Doroshow 1983; Sinha et al., 1987; Taatjes et al., 1997]. Similar to many xenobiotics, doxorubicin is known to initiate multiple cellular biochemical events and to target mitochondria [Taatjes et al., 1997; Nilsson et al., 1989]. Relevant to our study, while ROI release is minimal when mitochondria remain coupled, mitochondrial release of ROI markedly increases under certain conditions inclusive of xenobiotic induced reductive stress [Dawson et al., 1993; Nilsson et al., 1989]. Our time course bioenergetic data suggest that in susceptible cells doxorubicin initially resulted in oxidative stress (lowered GSH levels, diminished energy generation) which progressed to chemically induced respiratory inhibition and a reductive

stress (manifest as a highly reduced redox state). Although the redox state is only reflective of the general cell metabolic state and is not, per se, an indicator of cell injury, preservation of the cellular redox balance is energy dependent [Kehrer and Lund, 1994]. Therefore, the end stage redox states and energy charges of the AIDS-KS cells suggest that doxorubicin uncoupled their electron transport systems, potentially by inactivating a vital component of the respiratory chain. This premise is supported by studies by Sarvazyan et al. [1995] which showed that doxorubicin inactivates cytochrome c in vitro. We surmise that the higher endogenous defenses in HIV^- donor fibroblasts reduced doxorubicin associated deleterious effects and allowed these strains to retain pathways necessary to generate energy and provide reducing equivalents at the appropriate sites.

Qualitatively, AIDS-KS cells appeared to accrue more doxorubicin associated damage at both the phase contrast and ultrastuctural microscopic levels. Our observations were in agreement with previous ultrastructural studies which also showed the most dramatic effects were noted at the plasma and mitochondrial membranes as well as within the mitochondria [Olson et al., 1974; Unverferth et al., 1981]. Not surprisingly, our observed mitochondrial ultrastructural perturbations, e.g., reduction in cristae, also correlated with diminished cellular energy status in doxorubicin susceptible cells [Mathews and van Holde, 1990]. However, we are aware that our biochemical and microscopic data only provide indirect evidence that the mitochondria are a targeted site of doxorubicin associated toxicity in AIDS-KS cells. Our laboratory is currently conducting mitochondrial functional analyses (MTT [thiazolyl blue] reduction and permeability transition assays) to temporally correlate doxorubicin associated mitochondrial perturbations with loss of cellular viability.

The increased plasma membrane damage observed at both the phase contrast and ultrastructural levels in the AIDS-KS cells implied cell membrane-doxorubicin interactions. Recent studies by Taatjes et al. [1997] which showed doxorubicin induced $OH\cdot$ radical formation primarily at extracellular sites, are supportive of our observations. Taajes et al. [1997] and Doroshow [1986] also demonstrated that exogenously administered (generally nonmembrane permeable) ROI degrading enzymes or metal chelators such as extracellular superoxide dismutase, catalase and desferal abrogated doxorubicin's toxic effects. Our results, which showed moderate cytoprotection exclusively in AIDS-KS cells when either desferioxamine or the $OH\cdot$ scavenger, DTMU, were included during doxorubicin challenge, are in agreement with these findings. Also, our findings that desferioxamine and DTMU were deleterious to the HIV-negative donor cells suggests that their inclusion impaired endogenous defenses. This premise is supported by studies by Koblin and Tomerson [1990] which show that in addition to its ROI scavenging properties, DTMU also modulates cellular protein chemistry thereby delaying enzyme turnover. Further, because desferioxamine only enters some mammalian cells [Doroshow, 1986], its nonbeneficial effects in HIV-negative donor cells may reflect either permeability differences or reduced extracellular redox chemistry in control cell strains. We speculate that the ROI generated as a consequence of membrane associated redox chemistry persist longer in the cytoprotective deficient AIDS-KS cells.

Our data, which show that redox stress occurs during doxorubicin associated AIDS-KS cytotoxicity, provides a biochemical rationale for doxorubicin's clinical efficacy in AIDS-KS treatment. Pertinent to the clinical situation, mucocutaneous AIDS-KS has features which make this disease amenable to treatment via local delivery of sustained release chemotherapeutic agents. Our laboratory is currently investigating the efficacy and relative selectivity of doxorubicin loaded biodegradable polymers which provide sustained drug release. Because it is well accepted that sustained oxidant stress is more deleterious to susceptible cells than transient episodes [Freeman and Crapo, 1982; Halliwell and Gutteridge, 1986; Kehrer and Lund, 1994], we are quietly optimistic regarding the potential of these chemotherapeutic formulations.

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