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Cultured AIDS-Related Kaposi's Sarcoma (AIDS-KS) Cells Demonstrate Impaired Bioenergetic Adaptation to Oxidant Challenge: Implication for Oxidant Stress in AIDS-KS Pathogenesis

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Abstract Despite its recognition as the most prevalent HIV associated cancer, speculation still abounds regarding the pathogenesis of AIDS-related Kaposi's sarcoma (AIDS-KS). However, it has been established that both cytokines, e.g. IL-6, and HIV-associated products, e.g., Tat, are integral in AIDS-KS cellular proliferation. Further, both experimental and clinical evidence is accumulating to link reactive oxygen intermediates (ROI) with both cytokine induction (primarily via nuclear factor-κB [NF-κB] dependent routes) as well as the subsequent cytokine, tumor necrosis factor α (TNFα) stimulation of HIV replication. Features of AIDS-KS patients, such as retention of phagocytes, presence of sustained immunostimulation, and a frequent history of KS lesions arising at traumatized sites, make oxidant stress a viable clinical factor in AIDS-KS development. Time course nucleotide profile analyses show that AIDS-KS cells have an inherent, statistically significant, biochemical deficit, even prior to oxidant stress, due to 1) a more glycolytic bioenergetic profile, resulting in lower levels of high energy phosphates (impairing capacity for glutathione [GSH] synthesis and DNA repair); 2) lower levels of NADPH (compromising the activities of GSSG reductase and peroxidase function of catalase); and 3) reduced levels of GSH (impeding both GSH peroxidase and GSH-S-transferases). Following exposure to physiologically relevant levels of H_2O_2, only the human microvascular endothelial cells (a putative AIDS-KS progenitor cell) responded with bioenergetic adaptations that reflected co-ordination of energy generating and cytoprotective pathways, e.g., retention of the cellular energy charge, increased NAD^+, and an accentuation of the ATP, NADPH, and total adenine nucleotide differences relative to AIDS-KS cells. Also, some of the AIDS-KS strains retained intracellular GSSG subsequent to oxidant challenge, inviting the formation of deleterious protein mixed disulfides. While the results of our study address some AIDS-KS issues, they also raise an etiological question, i.e., Does the inability to tolerate oxidant stress arise in conjunction with AIDS-KS neoplastic development, or is it pre-existing in the population at risk? Regardless, use of antioxidant therapy (low risk/potentially high benefit) in both the "at risk" population as well as in those individuals with active disease may prove a useful preventative and/or treatment modality.

Key words: reactive oxygen intermediates, nucleotides, glutathione, redox state, energy charge, DNA damage, apoptosis

AIDS-related Kaposi's sarcoma (AIDS-KS) is both an AIDS defining disease, and the most common HIV associated malignancy [1,35,41]. Although the patient demographics of AIDS-KS have been delineated (the vast majority of AIDS-KS individuals is comprised of men in the homosexual/bisexual risk group) the contribution of as yet unidentified cofactors responsible for its development are still speculative [3,41]. While epidemiological data suggest sexually transmissible agent(s) [3,41], in vitro experimental results have implicated cytokines, e.g., IL-6, as well as other HIV associated products, e.g., the HIV *tat* gene product, Tat, in the promotion of growth of AIDS-KS cells [7,8,28,29,36]. Data presented in this manuscript, in conjunction with previous publications, suggest that susceptibility to oxidant stress is a cofactor in AIDS-KS pathogenesis.

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Oxidant stress could promote an environment conducive for AIDS-KS development by several routes [17,32,40]. Recent data has shown that reactive oxygen intermediates (ROI) are implicated in the activation of a multifaceted transcriptional activator, nuclear factor-κB (NF-κB) [39,40]. Intracellular conditions which either increase the levels of ROI, or decrease intracellular thiol levels, promote the activation of NF-κB [39,40]. Because of its regulatory role in the induction of gene expression, one of the many results of NF-κB activation would be enhanced cellular cytokine production, creating the opportunity for an autologous growth loop [19]. Further, oxidant challenge with H_2O_2 has been demonstrated to induce NF-κB associated cytokine production by a route which is inhibited by the hydroxyl radical scavenger, dimethyl sulfoxide [6]. Additional studies, which are supported by clinical trials using antioxidants, have shown that increases in intracellular thiol content inhibit tumor necrosis factor α (TNF-α) associated stimulation of HIV replication, thereby suggesting that TNF-α modulates some of its effects via an ROI dependent route [40]. During AIDS disease progression, patients' sera levels of TNF-α, and subsequently ROI levels, increase in parallel with patient susceptibility to neoplasia [11,33]. Also, despite their acquired $CD4^+$ lymphopenia, HIV^+ individuals retain their neutrophils and macrophages [5]. Therefore, activated phagocytes, especially HIV infected macrophages, can play a major role in the development of AIDS-KS by their production of established KS growth promoters such as IL-6 and Tat, as well as oxidative burst generated ROI [5,8,10].

We have recently demonstrated that, relative to either human fibroblasts or human microvascular endothelial cells (HMECs), cultured AIDS-KS cells are susceptible to oxidant stress due to significantly lower levels of catalase, as well as a tripeptide integral in cytoprotection, glutathione (GSH) [21]. In this current study, we conducted a time course analysis of cellular bioenergetic and GSH responses during, and subsequent to, introduction of physiologically relevant levels of H_2O_2. We determined that relative to HMECs (a putative AIDS-KS progenitor cell) AIDS-KS cells have a biochemical deficit in their ability to respond to environmental perturbations. Following H_2O_2 exposure, HMECs retained a nucleotide profile conducive to cytoprotection that was characterized by abundant levels of high energy phosphates, reducing equivalents in the form of GSH and NADPH, NAD^+, and total adenine nucleotides. In contrast, following H_2O_2 challenge, AIDS-KS cells possessed a bioenergetic profile suggestive of failure to co-ordinate energy generating and cytoprotective pathways, e.g., trends toward a concurrent reduction of the cellular redox state and a decreased cellular energy charge. Studies to evaluate intracellular GSH status revealed that AIDS-KS cells not only possess significantly lower levels of GSH, but that cellular capacity to reduce GSSG following oxidant challenge is compromised in some AIDS-KS cell strains.

Due to the extensive interdependency among ROI generation, autologous cytokine production, and viral replication, it is difficult to precisely identify the role that each component plays in AIDS-related disease progression. However, our findings, which demonstrate an inherent deficit in AIDS-KS cytoprotection, in conjunction with clinical data, provide compelling evidence that oxidant stress is associated with AIDS-KS pathogenesis.

MATERIALS AND METHODS

Isolation and Culture of AIDS-KS Cells

HIV+ patients 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 excisional biopsy of the suspected KS lesion, an examination was conducted to determine the extent and clinical presentation of the patients' 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 [21]. For these experiments, the KS cells were cultured on human fibronectin coated flasks, and grown in "Complete" medium, which consisted of: M-199 (GIBCO, Grand Island, NY), supplemented with 15 mM HEPES, 2.9 μg/ml l-glutamine, 1.1 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.

Isolation and Culture of Human Microvascular Endothelial Cells (HMECs)

HMECs were isolated as previously described [22,24], and cultured under the same conditions as the AIDS-KS cells.

Evaluation of Cellular Response During H_2O_2 Challenge

To evaluate cellular response to physiologically relevant levels of ROI, cultures of HMECs and KS cells were exposed to exogenous H_2O_2, at a concentration of 200 μM [10,13,34]. Cellular aliquots were taken during harvest for enumeration and viability assessment (via trypan blue exclusion) during the oxidant challenge experiments.

Proliferative cultures of HMECs and KS cells were trypsinized, and the cells plated at a density of $4.5 \times 10^6/150$ cm^2 in a M-199 base medium supplemented with 15 mM HEPES, 1-glutamine (2.9 μg/ml), Na pyruvate (1.1 μg/ml), and 2% heat inactivated fetal bovine serum, and the cultures incubated for 12 h at 37°C, 5% CO_2. Reduced serum levels were used to decrease overnight mitotic activity (retaining constant cell numbers), but still incorporating some serum cellular attachment factors. The morning of the assay, the cultures were then gently rinsed with room temperature PBS, and the medium replaced with M-199 medium containing HEPES, 1-glutamine, Na pyruvate, with 200 μM H_2O_2 (challenged cultures) or without H_2O_2 (control cultures). Photographs were taken, on a 37°C temperature controlled stage, prior to, during, and following recovery from, the addition of H_2O_2.

Timed Nucleotide Harvests to Evaluate Bioenergetic Adaptation to Oxidant Challenge

To assess both the short-term responses, in addition to the recovery capacities, of the cellular bioenergetic profiles during oxidant challenge, cultures were harvested for nucleotide analyses at five specific time points: control (nucleotide levels following overnight incubation in 2% FBS medium), after a 5 min and 1 h challenge with 200 μM H_2O_2, and after a 1 h 200 μM H_2O_2 challenge followed by either a 24 h or 7 day recovery in "Complete" medium.

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].

Cells were harvested, washed twice with PBS, and resuspended in 500 μl of PBS-0.02% EDTA. Cellular extractions were conducted in chilled microfuge tubes that contained a bottom layer of 100 μl of 2M perchloric acid (PCA), with an overlying organic layer comprised of 500 μl of 93% bromododecane (BDD), 7% dodecane (DD). The cell samples were gently layered over the chilled BDD/DD/PCA, centrifuged for 1 min at 14,000 rpm in a refrigerated (4°C) microcentrifuge, and the top aqueous phase removed with a pasteur pipette. A cotton swab was used to remove the BDD/DD, and clean the sides of the microfuge tube. The cellular pellet was dispersed by addition of 200 μl of ice-cold H_2O, followed by vortexing. The resuspended cellular extracts were kept on ice for 10 min, and then centrifuged at 14,000 rpm, 4°C, for 1 min. The protein pellets were retained for the Lowry assay, while the PCA extracts were transferred to another cold microfuge tube, and 500 μl of freshly prepared, ice cold 4:1 freon:triocytlamine added to neutralize the sample by extraction of the PCA. The samples were vortexed vigorously for 30 s, and then centrifuged for 1 min, 4°C, 14,000 rpm. The top, clear aqueous layer was removed, and stored at −20°C until injected onto the HPLC column.

Chromatography

Adenine, nicotinamide nucleotides, and respective nucleosides were separated on a Partisil 10 SAX column (Whatman, Hillsboro, OR) using a phosphate and pH gradient [14]. Buffer A consisted of 0.01 M H_3PO_4, pH 2.65; buffer B was 0.75 M KH_2PO_4, pH 4.5, with detection at 254 nm. Because reduced nicotinamide nucleotides undergo degradation during acid extraction, PCA-exposed NADH and NADPH standards were used to determine the position of the reduced nicotinamide nucleotide degradatory fragments. Notably, both the NADH and NADPH peaks separated at distinct sites from other peaks, and the area under the NADH and NADPH peaks remained constant during varying lengths of PCA exposure.

In many of the chromatograms, the UTP and CTP peaks co-eluted. Therefore, the levels of these high energy phosphates are expressed as UTP and CTP. Results were expressed as nmol/mg protein.

Protein Determination

Cellular protein levels were determined by the Lowry method, using bovine gamma globulins as the standard protein [20]. To remove any residual BDD/DD, the nucleotide protein samples were washed twice in acetone. To deter-

mine if the BDD/DD separation and the acetone washing affected the sample protein content, or its optical density, bovine gamma globulin standards were centrifuged through the BDD/DD, and washed twice with acetone. Comparison of the optical densities obtained between treated and nontreated bovine gamma globulin standards demonstrated that the acetone wash did not affect optical densities, nor did it result in protein loss.

Response of Cellular Total Glutathione Status to H_2O_2 Challenge

Because cellular thiol redox perturbations occur rapidly [27,38], cultures were harvested to monitor GSH status at the two earliest nucleotide-corresponding time points, i.e., following a 5 min and 1 h exposure to exogenous H_2O_2. The response of intracellular GSH levels to oxidant challenge was determined by evaluation of the total GSH (GSH plus GSSG), as well as the percentage of intracellular GSH present as the disulfide, GSSG.

Cultures of human microvascular endothelial and AIDS-KS cells that had been incubated overnight in base medium-2% FBS were gently rinsed with room temperature PBS. The base medium-2% FBS was replaced by base medium only (controls), or base medium supplemented with 200 μM H_2O_2. Cellular alteration in GSH status during H_2O_2 challenge was then evaluated at 5 min after the introduction of H_2O_2, and following a 1 h H_2O_2 challenge at 37°C, 5% CO_2, while the control cultures were incubated for 1 h in base medium.

After harvesting, the cell pellet was resuspended in PBS-EDTA to a volume of 0.5 ml (GSH samples), or 0.4 ml (GSSG samples). For the GSH samples, the proteins were then precipitated by addition of an equal volume of 2M PCA, and the samples stored at −20°C. Twelve hours or less prior to the GSH assay, the supernates were neutralized to pH 6.02 (using KOH and 0.4 M morpholineethanesulfonic acid [MES], Sigma Chemical Co., St. Louis, MO), and frozen overnight. For the GSSG samples, 100 μl of 0.2 M N-ethylmalemide (N-EM), and 500 μl of 2 M PCA were added. Prior to assay, the GSSG samples were neutralized by addition of 1 ml of 1.3 M K_2HPO_4, stirred for 30 min, centrifuged, and kept on ice for 5 min. The supernates were then treated 5 times with ice-cold, H_2O saturated ethyl acetate to extract the N-EM, followed by a stream of nitrogen gas to remove the remaining ethyl acetate. The GSSG samples were then frozen at −20°C, and assayed within 12 h.

Cellular levels of GSH and GSSG were determined according to the method of Eyer and Podradsky [1986]. NADPH, GSH, GSSG, glutathione reductase (type IV), and DTNB were obtained from Sigma. The enzymatic kinetics in the Eyer assay have been modified so that the glutathione reductase catalyzed step is no longer rate limiting. Therefore, this assay is much less susceptible to any endogenous, intrasample glutathione reductase inhibitors [30].

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 s/in. Sample concentrations (nmol/ml) were determined by comparison with a 10 point (GSH, range 0.2 to 2 nmol/ml) or 7 point (GSSG, range 0.125 to 1.5 nmol/ml) standard curve conducted concurrently. For control purposes, the GSSG standards were also extracted with H_2O saturated ethyl acetate. The results are expressed as nmol GSH or GSSG/mg protein.

RESULTS

Results obtained from this current, as well as our previous studies [2,21–23], indicate that the HMEC and AIDS-KS strains adapt favorably to growth in a "Complete" medium, which notably does not contain any conditioned medium from retrovirally infected cells. Evidence of positive cellular adaptations, common to both the HMEC and AIDS-KS strains, to in vitro culture conditions include high mitotic indices during proliferative growth, cell cycle completion times between 24 and 36 h, possession of an energy charge of ≥ 0.90 (e.c. = [ATP] + ½[ADP]/[ATP] + [ADP] + [AMP]) indicative of ongoing, active oxidative metabolism, and retention of a constant, strain specific, cellular phenotype. Consistent with their endothelial origin, cultured HMECs possessed a uniform, cobblestone morphology [21]. In contrast, a greater degree of phenotypic heterogeneity (primarily variations on a spindled morphology) was noted in the AIDS-KS cultures [21]. Also, unlike the growth properties of the HMECs, the AIDS-KS cells [21] showed growth characteristics consistent with a transformed phenotype—a lack of contact inhibition, loss of anchorage dependence, and formation of cellular foci in conjunction with increased intercellular adhesion [4,23,31].

In order to reduce overnight mitotic activity and maintain constant cell numbers prior to the H_2O_2 challenge experiments, the AIDS-KS and HMEC strains were plated in a base medium that contained markedly reduced serum (2% FBS) concentrations. As would be anticipated, both culture groups responded to the nutrient reduction by a marked decrease in mitotic activity, while some of the AIDS-KS cultures showed an increased tendency toward foci formation/cellular aggregates. However, cellular plating efficiency, as estimated by cell counts and numbers of nonadherent cells, was not adversely affected by use of this reduced nutrient medium.

Previously, we have established that cultured AIDS-KS cells are susceptible to oxidant challenge due to impaired cytoprotective capabilities [21]. Conceivably, H_2O_2 exposure of these cytoprotective deficient AIDS-KS cells could result in overall culture apoptosis, thereby preventing the transmittal of any ROI mediated mutations and negating the opportunity for cellular transformation. Therefore, to document retention of cellular capacity for a return to proliferative growth, cultures were given a 7 day recovery period in "Complete" medium following H_2O_2 challenge. Also, to ascertain whether H_2O_2 exposure impairs AIDS-KS cellular capacity for sustained proliferation, some of the 7 day recovery AIDS-KS cultures were trypsinized and passed for 4 additional population doubling levels. As a component of the oxidant challenge experiments (Table I), cellular aliquots were taken for cell counts and viability assessment. Several uniform findings are present in this enumeration/viability data. Following the overnight incubation in the "base medium" + 2% FBS, both the HMEC and AIDS-KS cultures showed an increase in cell number over the initial plating densities of 4.5×10^6 cells. Cellular viability for both the HMEC and the AIDS-KS cells was routinely $\geq 98\%$ during non-H_2O_2 exposed conditions. During the actual H_2O_2 exposure, both the HMECs and AIDS-KS cells showed a frank cell loss (approximately 32% in AIDS-KS cultures, 17% in HMECs); however, the viability of the surviving cells remained $\geq 95\%$. Also, by the 7 day recovery harvest, both the HMEC and AIDS-KS cultures had re-entered the cell cycle, and had undergone over one population doubling. Furthermore, the post-H_2O_2 recovery AIDS-KS cultures continued to proliferate following trypsinization and subsequent passage.

However, distinct differences are also apparent in this cell enumeration data. First, the HMEC cultures sustained a statistically significant lower overall cell loss during H_2O_2 challenge ($P < 0.001$ at the 5 min harvest, and $P < 0.01$ at the 1 h harvest, Student's 2 tailed t-test). Although the HMECs showed signifi-

TABLE I. Assessment of Cellular Adaptation to Oxidant Challenge: Total Cell Numbers and Viability†

Culture		Control	Exposure		Recovery	
			5 min	1 h	24 h	7 day
AIDS-KS	Viability (%)	≥95%	≥95%	≥95%	≥98%	≥98%
AIDS-KS	Cell count $\times 10^6 \pm$ SD	4.76 + 0.47 n = 5	3.22 ± 0.41 n = 10***	3.29 ± 0.65 n = 8**	3.78 ± 0.31 n = 5**	8.08 ± 0.59 n = 6**
HMECs	Viability (%)	≥98%	≥96%	≥95%	≥98%	≥98%
HMECs	Cell count $\times 10^6 \pm$ SD	5.29 + 0.26 n = 5	4.47 ± 0.76 n = 10***	4.39 ± 0.79 n = 10**	4.62 ± 0.20 n = 5**	9.88 ± 0.67 n = 5**

†Cultured AIDS-related Kaposi's sarcoma (AIDS-KS) cells and human microvascular endothelial cells (HMECs) were seated at a density of $4.5 \times 10^6/150$ cm^2 in "base medium" (M-199 supplemented with 15 mM HEPES, 2.9 μg/ml l-glutamine, 1.1 μg/ml Na pyruvate) plus 2% heat inactivated fetal bovine serum, and incubated overnight at 37°C, 5% CO_2. Hemocytometer cell counts and % viability (assessed by trypan blue exclusion) were conducted on aliquots obtained at specific time points: control (overnight incubation in base medium-2% FBS), following a 5 min and 1 h challenge with 200 μM H_2O_2 (conducted in "base medium"), and after a 1 h H_2O_2 challenge, followed by a 24 h and a 7 day recovery in "Complete" medium ("base medium" supplemented with 150 μg/ml endothelial cell growth supplement; 90 μg/ml Na heparin; 15% heat inactivated fetal bovine serum; 5% heat inactivated, pooled, male human serum). Cell counts are expressed as mean values $\times 10^6 \pm$ S.D.; viability as % of cellular population that excluded trypan blue. Statistical analyses for comparison of the AIDS-KS versus HMEC cell number differences at the same harvest time point were conducted using the two-tailed Students t-test.

**$P < 0.01$.

***$P < 0.001$.

cantly higher overall cell numbers relative to the AIDS-KS cultures at the 24 h and 7 day recoveries, this data reflects the greater AIDS-KS cell losses during H_2O_2 challenge, and is not indicative of an AIDS-KS delay in return to proliferative growth. At the 24 h and 7 day recoveries, the AIDS-KS cultures showed 1.17- and 2.50-fold respective increases over their lowest cell numbers, seen at 5 min after H_2O_2 challenge. Comparable, but slightly lower cell number increases, 1.05- and 2.25-fold (relative to population low at 1 h H_2O_2 incubation), were demonstrated by HMECs.

Cellular response to exogenous H_2O_2 was dictated by both cell position in the flask and cellular endogenous cytoprotective capabilities. As a component of their in vitro growth characteristics, both the HMEC and AIDS-KS cultures showed increased cellular density at the central portion of the flask, with peripheral cellular density remaining lower until culture confluence. In addition, individual cellular responses to H_2O_2 challenge were found to be both time and cell density dependent (Fig. 1). Overt cell lysis occurred rapidly (within seconds to minutes of H_2O_2 addition), and was more severe at the less cell-dense flask peripheries in both culture groups. More subtle cellular perturbations, e.g., increased stress fibers, alterations in cell shape, decrease in cell size, and increased cytoplasmic blebbing, occurred over the duration of the one hour H_2O_2 challenge. Although both the HMEC and AIDS-KS cultures displayed these H_2O_2 induced changes, the perturbations were more extensive in the AIDS-KS strains. Further, while morphologically altered cells were absent from the HMEC cultures at the 24 h and 7 day recovery points, the AIDS-KS cultures retained subpopulations of morphologically altered cells at both recovery times. In both the HMEC and the AIDS-KS cultures, mitotic figures were noted within 24 h after H_2O_2 challenge (Fig. 1). However, while the HMECs migrated readily into the zones of former cell destruction, the AIDS-KS cells displayed more restricted migration. As a result, at the 7 day harvest, re-establishment of a cellular monolayer was commonly seen in the HMEC cultures, whereas the AIDS-KS cultures frequently contained cellular aggregates juxtaposed to zones devoid of cells (Fig. 1).

The HPLC nucleotide analyses showed several findings which were common to both the HMEC and AIDS-KS cultures (Table II). At all nucleotide harvest points, ATP was the primary high energy phosphate, and NAD^+/NADH represented the primary redox pair. Within the individual culture group (HMEC or AIDS-KS), oxidant exposure did not result in significant perturbation of nucleotide/nucleoside components, e.g., HMEC GTP levels remained fairly constant over the experimental time course. Recovery of oxidative metabolism following H_2O_2 exposure, as indicated by energy charges > 0.90, was common to both the HMEC and AIDS-KS cultures at the 24 h and 7 day harvests. Variations in strain sensitivity to serum deprivation, as reflected by control ATP levels, were found in both the AIDS-KS and HMECs. Notably, those cultures that were most "serum deprivation sensitive," demonstrated that they were not effete by their rebound of ATP levels and continued proliferation after reintroduction of "Complete" medium.

Fluctuations in the redox poise [ratio $NAD(P)H/NAD(P)^+$] and energy charge occurred during ongoing H_2O_2 exposure. At the 5 min and 1 h H_2O_2 exposure/harvest time points, the AIDS-KS cultures showed trends toward an energy charge decrease, in conjunction with reduction of the cellular redox poise. In contrast,

Fig. 1. Cellular responses to H_2O_2 challenge. Cultures of human microvascular endothelial cells (HMECs) and AIDS-related Kaposi's sarcoma cells (AIDS-KS) cells were plated at a density of 4.5×10^6 cells/150 cm^2 in a low serum medium (M-199 supplemented with 2% heat-inactivated fetal bovine serum, 15 mM HEPES, 1-glutamine (2.9 μg/ml), Na pyruvate (1.1 μg/ml)), and incubated overnight at 37°C, 5% CO_2. The cultures were gently rinsed with room temperature phosphate-buffered saline, and the medium replaced with the M-199 base supplemented with 1-glutamine, Na pyruvate, HEPES, which also contained 200 μM H_2O_2. During H_2O_2 challenge, both the HMEC (**A**) and the AIDS-KS (**B**) cultures showed a zonal component to their cell loss, which was most dramatic at the less densely packed flack peripheries. After a 24 h recovery in "Complete" medium (M-199 supplemented with 15 mM HEPES, 90 μg/ml sodium heparin, 150 μg/ml endothelial cell growth supplement, 15% heat-inactivated fetal bovine serum, 5% heat-inactivated, pooled human male serum), both the HMECs (**C**), and the AIDS-KS (**D**) cultures showed a return to proliferative growth as can be appreciated by ongoing mitotic activity. After the 7 day recovery, some HMEC cultures (**E**) still showed migration into, and proliferation at, zones of former cell destruction. In contrast, the AIDS-KS cells at the 7 day recovery (**F**) displayed restricted migration into the wounded regions, and demonstrated preferred formation of cellular aggregates/foci. Depicted just inferior to the center in (F) is a retained, abnormal AIDS-KS cell displaying perturbations consistent with cytoskeletal disruption. Photomicrographs: ×10 image scale (A,B), ×25 image scale (C,D), ×50 image scale (E,F).

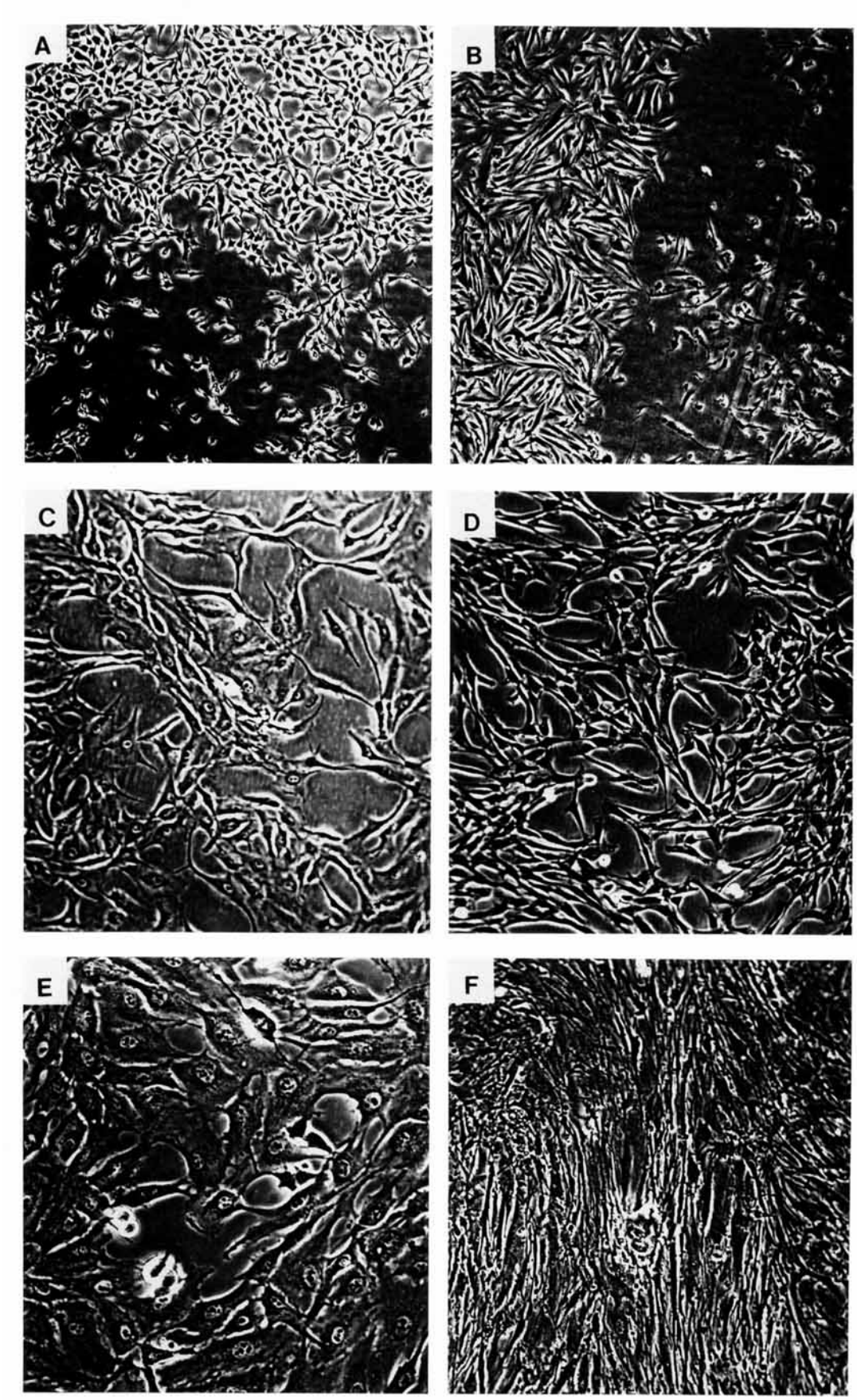

Figure 1.

TABLE II. HPLC Nucleotide Analyses: Timed Nucleotide Harvests to Evaluate Bioenergetic Adaptation to Oxidant Challenge

		Exposure		Recovery	
Culture	Control	5 min	1 h	24 h	7 day
A. HPLC Nucleotide profiles of AIDS-related kaposi's sarcoma cells					
AMP	0.56 ± 0.54	2.64 ± 3.87	0.91 ± 1.03	0.28 ± 0.30	0.56 ± 0.15
ADP	1.76 ± 1.40	3.56 ± 3.99	5.19 ± 5.97	2.08 ± 1.90	1.24 ± 0.20
ATP	15.85 ± 12.38*	16.30 ± 10.11*	21.48 ± 12.50**	18.26 ± 11.84***	18.11 ± 3.49*****
Total	18.17 ± 12.37*	22.49 ± 11.90*	27.57 ± 13.70**	20.63 ± 13.82***	19.91 ± 3.69*****
NAD+	5.98 ± 2.21	6.25 ± 4.27	4.49 ± 2.32**	6.16 ± 2.85**	6.57 ± 0.91*****
NADH	3.20 ± 0.98	3.77 ± 1.06	3.44 ± 1.59	3.95 ± 2.44	3.30 ± 0.48*****
Total	9.18 ± 2.93	9.69 ± 4.93	7.61 ± 3.47	10.09 ± 5.10	9.87 ± 1.31*****
NADP+	0.29 ± 0.20	0.34 ± 0.26	0.39 ± 0.30	0.27 ± 0.19	0.16 ± 0.04
NADPH	0.10 ± 0.10*	0.20 ± 0.10*	0.15 ± 0.12*	0.14 ± 0.05**	0.21 ± 0.10****
Total	0.39 ± 0.20	0.53 ± 0.31	0.55 ± 0.40	0.40 ± 0.21	0.37 ± 0.10
GTP	2.52 ± 1.99*	3.13 ± 1.93**	3.63 ± 2.23**	2.86 ± 1.75***	3.71 ± 0.55*****
UTP&CTP	3.66 ± 1.56**	3.95 ± 2.11***	5.19 ± 1.97**	6.43 ± 2.85	4.91 ± 0.78*****
Energy charge	0.91 ± 0.07	0.80 ± 0.24	0.88 ± 0.11	0.94 ± 0.003	0.94 ± 0.02
Redox poise	0.54 ± 0.09	0.74 ± 0.29	0.82 ± 0.41	0.64 ± 0.15	0.52 ± 0.05
B. HPLC nucleotide profiles of human microvascular endothelial cells					
AMP	0.62 ± 0.77	0.48 ± 0.29	1.11 ± 1.70	0.63 ± 0.73	0.33 ± 0.15
ADP	2.47 ± 1.50	1.93 ± 0.46	2.49 ± 1.29	1.51 ± 0.07	3.41 ± 2.57
ATP	43.76 ± 15.80*	41.45 ± 7.96*	43.06 ± 8.99**	48.79 ± 3.49***	41.46 ± 3.64*****
Total	46.84 ± 16.74*	43.47 ± 8.20*	46.67 ± 7.10**	50.93 ± 3.22***	45.21 ± 6.20*****
NAD+	10.41 ± 4.59	12.41 ± 1.58	12.11 ± 3.82**	11.34 ± 1.29**	14.35 ± 1.70*****
NADH	5.77 ± 2.68	4.36 ± 0.52	4.29 ± 0.84	4.40 ± 0.13	7.48 ± 0.57*****
Total	16.18 ± 6.84	16.78 ± 1.56	16.41 ± 4.46	15.74 ± 1.38	22.03 ± 2.18*****
NADP+	0.25 ± 0.17	0.15 ± 0.09	0.11 ± 0.09	0.21 ± 0.03	0.24 ± 0.18
NADPH	0.34 ± 0.14*	0.31 ± 0.06*	0.42 ± 0.23*	0.27 ± 0.04**	0.48 ± 0.18****
Total	0.59 ± 0.29	0.46 ± 0.15	0.54 ± 0.19	0.48 ± 0.05	0.73 ± 0.36
GTP	6.62 ± 2.27*	6.21 ± 0.97**	6.32 ± 1.16**	7.39 ± 0.74***	7.71 ± 0.60*****
UTP&CTP	8.48 ± 3.99**	8.15 ± 1.11***	8.07 ± 1.76**	9.41 ± 1.72	8.75 ± 0.47*****
Energy charge	0.96 ± 0.03	0.97 ± 0.01	0.94 ± 0.04	0.97 ± 0.01	0.96 ± 0.03
Redox poise	0.59 ± 0.18	0.38 ± 0.07	0.41 ± 0.11	0.48 ± 0.04	0.55 ± 0.04

Cultured AIDS-related Kaposi's sarcoma cells (AIDS-KS) and human microvascular endothelial cells (HMECs) were seated at a density of $4.5 \times 10^6/150$ cm^2 in "base medium" (M-199 supplemented with 15 mM HEPES, 2.9 μg/ml l-glutamine, 1.1 μg/ml Na pyruvate) plus 2% heat inactivated fetal bovine serum, and incubated overnight at 37°C, 5% CO_2. Cultures were rinsed with room temperature phosphate buffered saline, followed by the introduction of "base medium" that contained 200 μM H_2O_2. Cells were harvested for HPLC nucleotide analyses at five specific time points: control (nucleotide levels following overnight incubation in 2% FBS medium), after a 5 min and 1 h challenge with 200 μM H_2O_2 (conducted in "base medium"), and after a 1 h 200 μM H_2O_2 challenge, subsequently followed by a 24 h and 7 day recovery in "Complete" medium (M-199 base medium supplemented with 15 mM HEPES, 90 μg/ml sodium heparin, 150 μg/ml endothelial cell growth supplement, 15% heat inactivated fetal bovine serum, and 5% pooled, heated inactivated human male serum). n = 5 for all time points for both the AIDS-KS cells and HMECs, with the exception of the 7 day recovery for the AIDS-KS cells, when n = 6. Levels expressed as nmol/mg protein + S.D. Statistical analyses were conducted, using the Mann Whitney U (2 tailed)-test, on nucleotide profiles of AIDS-KS vs. human microvascular endothelial cells during harvest at the same time point. Redox poise = ratio NAD(P)H/NAD(P)$^+$.

*$P < 0.032$.
**$P < 0.016$.
***$P < 0.0008$.
****$P < 0.018$.
*****$P < 0.004$.

at these same time points, the HMEC cultures showed trends toward redox poise oxidation, while maintaining their cellular energy charge. Statistical analyses of the nucleotide profiles were conducted, using the two-tailed Mann Whitney U-test, on HMEC vs. AIDS-KS nucleotide components during harvest at the same time point. Some consistent (i.e., HMEC vs. AIDS-KS differences present prior to H_2O_2 challenge, and sustained throughout the experimental time

course) statistically significant differences were noted in the HPLC profiles, which were that 1) ATP levels were always higher in HMECs; 2) ATP differences in HMECs vs. AIDS-KS cells were accentuated after H_2O_2 exposure; 3) the total adenine nucleotide pool was higher in the HMECs, with this difference also being accentuated following H_2O_2 challenge; 4) NADPH levels were always greater in HMECs; and 5) GTP levels were consistently higher in HMECs. Also, with the exception of the 24 h recovery time point, UTP and CTP levels were significantly higher in the HMEC cultures. Other HMEC vs. AIDS-KS significant differences arose following H_2O_2 challenge. The HMECs' levels of NAD^+ were increased at the 1 h exposure time point, and remained significantly higher at both the 24 and 7 day harvest times. Also, at the 7 day harvest, the HMECs possessed higher levels of the redox pair NAD^+/NADH. Finally, throughout the course of the experiment, the energy charge of the HMEC cultures remained > 0.90, while the mean energy charge of the AIDS-KS cultures decreased below 0.90 during actual H_2O_2 exposure.

Evaluation of the cellular GSH status revealed that statistically significant differences exist between HMEC and AIDS-KS GSH levels at all harvest points, i.e., control, 5 min and 1 h after H_2O_2 exposure, and during proliferative growth (Fig. 2). Whereas AIDS-KS GSH levels remained constant, albeit low, over the experimental time course, the HMEC cultures responded to serum deprivation with a GSH decrease. These HMEC GSH differences were only significant in comparison of the control vs. proliferative HMEC GSH levels. Further, while two AIDS-KS strains retained detectable intracellular levels of the disulfide, GSSG, at the 5 min and 1 h harvests (GSSG levels represented 3.5% of total cellular GSH), none of the HMEC cultures contained any detectable intracellular GSSG levels. Neither the HMEC or the AIDS-KS cultures contained any detectable intracellular GSSG during proliferative growth.

DISCUSSION

This is the first investigation to monitor HMEC and AIDS-KS nucleotide profiles and cellular GSH status during ongoing challenge with physiologically relevant levels of H_2O_2. Features of AIDS-KS patients, such as retention of their phagocytic cells, presence of sustained immunostimulation, and frequent history of a Koebner phenomenon (local trauma followed by subsequent development of a KS lesion at the injured site) provide clinical relevance to the investigation of H_2O_2-cellular interactions [35,41].

A constant finding in both the HMEC and AIDS-KS cell enumeration data was retention of high viability, even during ongoing H_2O_2 challenge. Our viability data likely reflects the mechanical stress necessary to harvest the cells. It is probable that any markedly damaged cell would be lost at this stage, resulting in maintenance of an overall high cellular viability. The increase in HMEC and AIDS-KS cell numbers during the overnight incubation in low nutrient medium may be accounted for by a residual serum stimulation effect, as well as the inclusion of cells that were already committed to cell cycle progression. In light of our earlier data [21], the finding that HMECs retained higher cell numbers after H_2O_2 challenge was not unanticipated. However, what was surprising was the rapid return of AIDS-KS cells to proliferative growth. This suggests that if DNA damage occurred a significant delay for DNA repair did not take place. Another HMEC/AIDS-KS culture group-associated difference was that only the HMECs showed restoration of a cellular monolayer. Due to their endothelial cell lineage, HMECs generate abundant extracellular matrix [22,24], thereby restoring the matrix at zones of former H_2O_2 mediated human fibronectin. Further, AIDS-KS cells showing changes associated with apoptosis, e.g., cell shrinkage, were retained at the 24 h and 7 day recovery points. Because entrance into apoptosis requires gene induction and de novo protein synthesis (which may be blocked by proto-oncogene products such as Bcl-2) [5], genetically damaged AIDS-KS cells may circumvent apoptosis and be retained in the proliferating cell pool. Ongoing studies are being conducted in our laboratory to assess AIDS-KS cellular capacity for both DNA repair and entrance into apoptosis.

While both the HMEC and AIDS-KS cultures sustained H_2O_2 mediated changes, more profound perturbations were noted in the AIDS-KS cultures. In addition to a direct toxic reaction, undegraded H_2O_2 can have a deleterious effect on 1) cellular proteins; 2) membrane lipids, resulting in lipid peroxidation; 3) cytosolic molecules dependent upon hemoproteins, e.g., catalase; 4) nucleic acids and DNA, resulting in mutations and strand scission; and 5) cellular

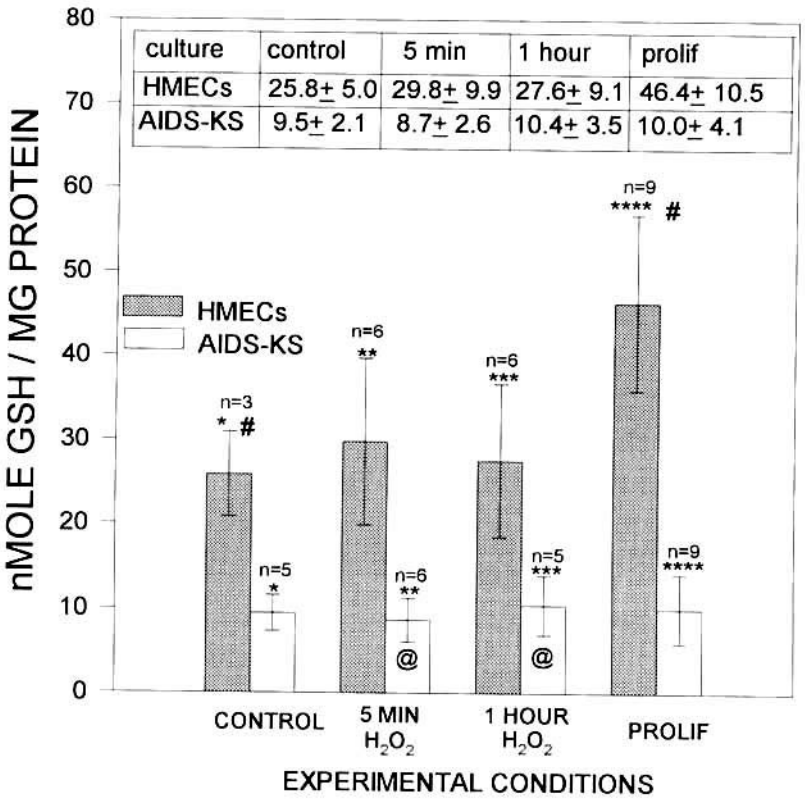

Fig. 2. Intracellular GSH levels of cultured AIDS-KS and human microvascular endothelial cells. Cultured AIDS-related Kaposi's sarcoma cells (AIDS-KS) and human microvascular endothelial cells (HMECs) were seated at a density of 4.5 × 10^6/150 cm^2 in "base medium" (M-199 supplemented with 15 mM HEPES, 2.9 μg/ml l-glutamine, 1.1 μg/ml Na pyruvate) plus 2% heat inactivated fetal bovine serum. Cells were harvested for GSH determination following overnight incubation in "base medium" plus 2% FBS; followed by a 1 h incubation in "base medium" (control); after a 5 min and 1 h H_2O_2 challenge (conducted in "base medium"); and during proliferative growth in "Complete" medium (M-199 "base medium" supplemented with 90 μg/ml Na heparin, 150 μg/ml endothelial cell growth supplement, 15% heat inactivated fetal bovine serum, 5% pooled, heat inactivated male human serum). Values are expressed as nmol total GSH/mg protein ± S.D. Statistical analyses were conducted using the Mann Whitney U (2 tailed)-test. *$P < 0.036$, **$P < 0.002$, ***$P < 0.008$, ****$P < 0.001$ AIDS-KS vs. HMEC GSH levels, #$P < 0.001$ HMEC control vs. proliferative GSH levels. @ = detectable levels of GSSG (comprising 3.5% of total GSH) found at harvest in 2 cultures.

bioenergetic state [16,18,37,38]. Further, residual H_2O_2 can markedly decrease the available ATP pool both by inhibition of a key glycolytic enzyme (glyceraldehyde-3-dehydrogenase), as well as by induction of ATP employing DNA repair enzymes [25,26].

While the HMEC GSH levels decreased during the overnight incubation in low nutrient medium, no medium related GSH differences were found in the AIDS-KS cultures. Because HMEC's GSH levels reflect cellular growth state [22], the HMEC's GSH decrease during serum deprivation likely reflects cellular entrance into a G0/quiescent growth state, thereby diminishing cellular need for GSH's contribution in DNA synthesis [15]. As a result of their significantly lower GSH levels, AIDS-KS cells are at a cytoprotective deficit; this problem is intensified in those AIDS-KS cultures that retained intracellular GSSG after H_2O_2 challenge. Intracellular GSSG promotes protein mixed disulfide formation, and may reflect either low GSSG reductase activity and/or decreased availability of the GSSG reductase cofactor, NADPH, in AIDS-KS cells [27].

Our data show distinct differences between HMEC and AIDS-KS cellular capacities to respond to the environmental stresses (serum deprivation and oxidant challenge) employed in this study. Because cellular ability to adapt to environmental perturbations is determined by induction of stress response genes [25,26], this data, in conjunction with our previous findings [21], imply that inherent, key deficiencies in cytoprotective abilities exist in AIDS-KS cells. Subsequent to serum deprivation, only the AIDS-KS cells became more glycolytic, thereby assuming

a biochemical profile reminiscent of the anaerobic phenotype shown by many tumor cells [31]. Notably, the AIDS-KS cells remained proliferative despite manifesting a cytoprotective deficient biochemical profile, therefore showing cellular growth kinetics that likely impact adversely upon the host.

During oxidative stress, three major cellular systems (mitochondrial, glycolytic, and plasma membrane oxidases), in conjunction with the NAD^+/NADH redox pair, must interact to maintain the cellular bioenergetic status [25,26]. AIDS-KS cells demonstrated compounding problems, as can be appreciated by their decreased energy charge and increased redox poise, during oxidant challenge. Cellular inability to sustain levels of NAD^+ not only inhibits glycolysis, resulting in loss of substrates for both the Kreb's cycle and the electron transport system [25,26], but also limits substrates necessary for DNA repair (NAD^+ and ATP). In addition to de novo NAD^+ synthesis, other cellular pathways, such as the malate shuttle and lactate dehydrogenase, are available to recover/regenerate NAD^+ [25,26]; these routes may be compromised in AIDS-KS cells. Furthermore, pyruvate dehydrogenase, and therefore glycolysis, is inhibited under conditions of fatty acid oxidation [25,26]. Due to their decreased capacities to withstand oxidant challenge, lipid peroxidation and its associated sequelae are likely occurring in AIDS-KS cells. Studies are ongoing in our laboratory to evaluate AIDS-KS and HMEC potential for lipid peroxidation during oxidant challenge.

In conclusion, we present results which demonstrate that cultured AIDS-KS cells show a deficit in their ability to biochemically respond in a cytoprotective fashion after exposure to physiologically relevant levels of H_2O_2. Our findings from both this current and previous AIDS-KS study [21], in conjunction with clinical data correlating sustained inflammatory states with neoplasia [42], as well as TNFα and ROI association with stimulation of HIV replication [17,32,33], implicate oxidant stress as a cofactor in AIDS-KS pathogenesis. A question that remains is whether an inherent susceptibility to oxidant stress is pre-existing in patients that develop AIDS-KS, or if intolerance to oxidants arises as a consequence of cellular transformation during AIDS-KS neoplastic development. Regardless, institution of antioxidant therapy (low risk/potentially high benefit) in both the population at risk to develop AIDS-KS, as well as in those individuals with active disease, may prove a useful preventative and/or treatment modality.

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Author's Note

Both this current study and our previous work reported in J Cell Biochem (1994) 56:568–581 employed a 200 μM [H_2O_2]. The [H_2O_2] in our 1994 article was incorrectly reported as 20 μM.

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