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Cultured AIDS-Related Kaposi's Sarcoma Cells Retain a Proliferative Bioenergetic Profile But Demonstrate Reduced Cytoprotective Capabilities

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Abstract Features of AIDS-related Kaposi's sarcoma (AIDS-KS), such as the multifocal presentation at mucosal and epidermal sites subjected to trauma, suggest that AIDS-KS is initially a reactive hyperplasia that subsequently progresses to a neoplasia. It is recognized that there is an association between sustained inflammatory states and the subsequent development of neoplasia (e.g., ulcerative colitis/colonic adenocarcinoma). Furthermore, patients who develop AIDS-KS experience both a constant immune stimulation due to sustained high levels of virus-induced cytokines and, because of a sparing effect on their phagocytic cells, retention of the phagocytic inflammatory response. A component of phagocytic activation is the initiation of the oxidative burst, resulting in the generation of reactive oxygen species (ROS), which can be mutagenic to host cells if released beyond the phagolysosome and not inactivated. Our results demonstrate that cultured AIDS-KS cells possess decreased cytoprotective capabilities. Relative to either dermal fibroblasts, or human microvascular endothelial cells (HMECs), AIDS-KS cells contained significantly lower levels of glutathione, a tripeptide integral in both cytoprotection and maintenance of cellular thiol status. While HMECs increased catalase activity during culture in the cytokine-rich KS milieu (control medium supplemented with conditioned medium from MOT, an HTLV II-infected cell line), AIDS-KS cells demonstrated reduced catalase function under these conditions. Furthermore, HMEC cultures showed an inherent biochemical responsiveness, by increasing catalase activity following exposure to exogenous H_2O_2. In contrast, the catalase activity of AIDS-KS cells decreased following H_2O_2 challenge. Our results show that an inherent deficiency in cellular cytoprotection is present in AIDS-KS cells and suggest that oxidant stress may function in the development and progression of AIDS-KS.

Key words: oxidant stress, nucleotides, glutathione, catalase, redox state, energy charge, reactive oxygen species

Kaposi's sarcoma (KS) is one of the myriad of diseases that affect AIDS patients [Armes, 1989; Roth et al., 1992; Roth, 1991; Tappero et al., 1993]. However, despite its name, evidence suggests that during its incipient stages, AIDS-KS is a hyperplasia [Armes, 1989; Roth et al., 1992; Roth, 1991; Tappero et al., 1993]. Unlike most malignancies, which establish a primary tumor prior to metastasizing, the lesions of AIDS-KS are frequently multifocal at onset [Armes, 1989; Roth et al., 1992; Roth, 1991; Tappero et al., 1993]. Furthermore, this multifocal lesional presentation at sites perfused by the microvasculature, suggests the contribution of a systemic, circulating component in AIDS-KS initiation [Armes, 1989; Roth et al., 1992; Roth, 1991; Tappero et al., 1993].

The internal milieu of patients that develop AIDS-KS is characterized by a constant immune stimulation due to sustained high levels of viral induced cytokines [Eversole, 1992; Miles, 1992; Rosenberg and Fauci, 1990]. In addition to the HIV virus, many AIDS-KS patients are co-infected with residual viruses, such as herpes simplex virus-1 and -2 (HSV-1 and -2), Epstein–Barr virus (EBV), cytomegalovirus (CMV), and hepatitis A and B [Eversole, 1992]. Notably, many patients that develop AIDS-KS relate a history of a Koebner type phenomenon in which local trauma is followed by the subsequent development of a KS lesion at the injured site [Roth

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et al., 1992; Tappero et al., 1993]. The injury-induced local inflammatory response, in conjunction with the ongoing constant immune stimulation, may initiate the cellular hyperplasia that results in a KS lesion. Once stimulated to proliferate, KS cells themselves generate many growth promoting cytokines, thereby initiating an autocrine growth pathway [Corbeil et al., 1991; Miles et al., 1990; Sturzl et al., 1992].

The association between sustained inflammatory states and the subsequent development of a neoplasia is not novel (e.g., Barrett's esophagitis/esophageal carcinoma, and ulcerative colitis/colonic adenocarcinoma) [Weitzman and Gordon, 1990]. Furthermore, although during disease progression, AIDS patients suffer a marked decrease in $CD4^+$ lymphocytes, there is generally a sparing effect on their macrophages and neutrophils [McCarthy, 1992]. Consequently, the hosts' phagocytic inflammatory response remains intact. A component of phagocytic activation is the initiation of a cellular oxidative burst, resulting in the generation of reactive oxygen species (ROS) [Weitzman and Gordon, 1990]. ROS, composed principally of peroxides and oxygen radicals (e.g., hydroxyl radical, OH^-), can be mutagenic to adjacent cells if released beyond the phagolysosome and not inactivated [Halliwell and Aruoma, 1991]. As a result, host cells have evolved a battery of cytoprotective capabilities to degrade ROS [Halliwell and Aruoma, 1991].

Reports imply an association between aberrant ROS degrading capabilities and the subsequent development of neoplasia; thereby linking sustained proinflammatory states, decreased cytoprotection, and cancer [reviewed in Weitzman and Gordon, 1990]. The results presented in this paper demonstrate that cells isolated from lesions of AIDS-KS are deficient in cellular cytoprotective capabilities. We suggest that this cellular deficiency results in ROS-associated alterations (e.g., perturbation in membrane lipids, DNA mutations) and is an instigating factor that accelerates lesional progression from hyperplasia to neoplasia.

Because of the recognized importance of KS in the HIV^+ population, many papers have been published on cultured AIDS-KS cells. However, much of the AIDS-KS literature has focused on determination of the histogenesis of the KS lesional cell, and the immunohistochemical properties of AIDS-KS cells, and has neglected the cellular biochemistry [4,24,29]. [Corbeil et al., 1991; Miles et al., 1990; Roth et al., 1992].

This paper presents results that begin to characterize AIDS-KS cells biochemically, and we investigate the contribution of the AIDS-KS milieu on these parameters. Three interrelated biochemical areas were evaluated: bioenergetic status (reflected in cellular growth characteristics, and adaptation to in vitro conditions), glutathione (GSH) levels (participation in maintenance of thiol status and cytoprotection), and catalase activity (degradation of H_2O_2). Because in previous findings we determined that there was a strong environmental influence on cellular bioenergetic status and GSH levels in a putative KS progenitor cell [Mallery et al., 1994], our AIDS-KS cells were cultured under two conditions: (1) an "AIDS-KS milieu," and (2) a control medium. Evaluation of cellular catalase activity was selected as the third biochemical parameter in this study. We targeted cellular cytoprotection against H_2O_2 for the following reasons. First, H_2O_2 is generated in appreciable quantities by activated phagocytes [Halliwell and Aruoma, 1991; Schraufstatter et al., 1985; Weitzman and Gordon, 1990]. Second, H_2O_2 is both membrane permeable, and, due to its lower reactivity, long-lived [Halliwell and Aruoma, 1991]. Intracellular H_2O_2 can be reduced to the complete carcinogen, hydroxy radical (OH^-), through Fenton chemistry when in the presence of reduced iron or copper [Halliwell and Aruoma, 1991]. Finally, cellular GSH status is related to catalase activity/H_2O_2 degradation by virtue of GSH peroxidase [Meister, 1988; Sandstrom and Marklund, 1990].

Cellular levels of GSH and catalase activity were also determined in two nontransformed cell strains-human microvascular endothelial cells (a putative KS progenitor cell), and human dermal fibroblasts. Our results demonstrate that, relative to either human microvascular endothelial cells (HMECs) or dermal fibroblasts, cultured AIDS-KS cells possess reduced cellular cytoprotective capabilities and imply that an inherent defeciency in cellular cytoprotection is an instigating factor that promotes the development of AIDS-KS.

MATERIALS AND METHODS

Isolation and Culture of AIDS-KS Cells

HIV^+ patients that had clinical lesions suggestive of KS were referred for participation in our 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, ten out of fourteen biopsies were microscopically confirmed by two Board certified Oral Pathologists as AIDS-KS. Seven KS cultures (obtained from a range of low- to high-grade KS lesions) have been established.

The KS tissue explant was meticulously dissected to remove any nonlesional tissue, and then treated by a selective enzymatic digest, which contained 2 mg/ml each of collagenases types Ia, II, and IV in 20 ml of 0.01% trypsin (collagenases type II and trypsin were purchased from Worthington Biochemical, Freehold, NJ; Ia and IV purchased from Sigma, St. Louis, MO), until a cloudy cellular suspension was obtained. Depending on the lesional site (e.g., palate vs. epidermis), the digest time ranged from 60 to 120 min. Prior to establishing KS primaries, preliminary studies were conducted on explants of histologically confirmed KS lesions to identify potentially problematic microbial contaminants in the KS biopsies. Our results showed that many of the AIDS patients carried staphylococcal strains that were either penicillinase producers, or methicillin resistant, and candidal strains that were fluconazole resistant. Therefore, an antibiotic/antifungal cocktail (amikacin sulfate, imipenem–cilastin, amphotericin B) was designed to combat these contaminants. KS primaries were established in a KS milieu (KSM), which consisted of M-199 (GIBCO), supplemented with 15 mM HEPES, sodium heparin (Sigma, 90 μg/ml), endothelial cell growth supplement (prepared in-house, from bovine brain, 150 μg/ml), 15% heat-inactivated fetal bovine serum (HYCLONE), 5% heat-inactivated pooled male human serum, 20% conditioned media derived from MOT, an HTLV-II-infected cell line [Mallery et al., 1994; Miles et al., 1990; Roth, et al., 1992], and amphotericin B (4 μg/ml), amikacin sulfate (50 μg/ml), and imipenem–cilastin sodium (25 μg/ml). The KS cells were plated onto human fibronectin (hFN) containing 5% conditioned MOT medium. Once the KS cultures were determined to be free of microbes, the antibiotic cocktail was removed from the KS medium.

To evaluate the effects of external environment on cellular growth regulation, KS strains were cultured in both KS milieu (*ksm*) and a control medium. The control medium (*con*) consisted of the same base medium (M-199), supplements, and serum as the KSM but did not contain any MOT-conditioned media in either the medium or hFN.

Isolation and Culture of Human Microvascular Endothelial Cells

Human microvascular endothelial cells (HMECs) were isolated from human eyes [Mallery et al., 1993]. According to Chung-Welch et al. [1989], these cells have been identified as endothelial in origin based on morphological characteristics, expression of specific adhesion receptor molecules, and spontaneous formation of microtubules in culture [Mallery et al., 1993]. These cultures have been screened for the absence of epithelial cytokeratins, using a pan-cytokeratin monoclonal antibody (Hybritech). The antibody HMB45 (DAKO) was used to determine the absence of any melanin-producing capability. Finally, cultures were shown to be non-pericyte by standard immunocytochemistry for smooth muscle-specific α actin (Sigma).

For these studies, HMECs were cultured in either the control medium (*con*), or the "KS milieu" (*ksm*), to determine whether there was an environmental effect on HMEC GSH levels or catalase activity.

Isolation and Culture of Human Fibroblasts

Human fibroblast cultures were established from full thickness skin biopsies that were removed during elective surgery. Fibroblast cultures were grown in a modification of Eagle's minimum essential medium (GIBCO, Grand Island, NY) supplemented with the addition of 1.5× essential amino acids, 1.5× vitamins, 2× nonessential amino acids, 1× *l*-glutamine, 10% heat-inactivated fetal bovine serum (HYCLONE) [17].

Determination of Cellular Bioenergetic Status and Redux Poise (Ratio NAD(P)H/NAD(P)$^+$)

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 phosphate-buffered saline (PBS), and resuspended in 500 μl of serum-free medium. Cellular extractions were conducted in chilled microfuge tubes containing a bottom layer of 100 μl of 2M perchloric

acid (PCA) with an overlying organic layer comprised of 500 μl of 93% bromododecane (BDD), and 7% dodecane (DD). The cell samples were gently layered over the ice-cold BDD/DD/PCA and centrifuged for 1 min at 14,000 rpm; the top aqueous phase was removed with a Pasteur pipette. A cotton swab was used to remove the BDD/DD and to clean the sides of the microtube. The cellular pellet was dispersed by addition of 200 μl ice-cold H_2O, followed by vigorous vortexing. The resuspended cellular extract were kept on ice for 10 min and then centrifuged for 1 min at 14,000 rpm. The PCA extract was then transferred to another cold microfuge tube, and 500 μl of freshly prepared ice-cold 4:1 freon/triocytlamine was added to extract the lipids and to neutralize the PCA. The sample was vortexed at maximum speed for 30 sec, and then centrifuged for 1 min at 14,000 rpm. The clear aqueous layer was removed, added to an equivalent volume of ice-cold H_2O, 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) using a phosphate and pH gradient [Geisbuhler et al., 1984]. Buffers were prepared fresh on the day of use, filtered through a 0.2-μM Millipore membrane filter, and degassed by helium spargling. 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 [Hohl et al., 1989].

On many of the cellular samples, 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 nucleotide/mg protein.

Protein Determination

Cellular protein levels were determined by the Lowry method, using bovine γ-globulins as the standard protein [Lowry et al., 1951]. To remove any residual BDD/DD, the nucleotide protein samples were washed twice in acetone, followed by centrifugation for 4 min at 14,000 rpm after each wash. To determine whether the BDD/DD separation and the acetone washing affected the sample protein content, or its optical density, bovine γ-globulin standards were centrifuged through BDD/DD and washed twice with acetone. Comparison of the optical densities obtained between treated and nontreated bovine γ-globulin standards demonstrated that the acetone wash did not affect optical densities, nor did it cause protein loss.

Determination of Total Cellular GSH

After harvesting and centrifugation, the cell pellet was resuspended to a volume of 0.5 ml in PBS–0.02% EDTA for number and viability (trypan blue) quantification. Proteins were precipitated via addition of an equal volume of 2M PCA. The samples were stored at −20°C. Twelve hours or less prior to assay, the supernates were neutralized to pH 6.02 (using 2M KOH and 0.4M morpholineethanesulfonic acid (MES) (Sigma Chemical Co., St. Louis, MO), and frozen overnight.

Cellular levels of total GSH (GSH and the disulfide, GSSG) were determined according to the method of Eyer and Podhradsky [Eyer and Podhradsky, 1986]. NADPH, GSH, glutathione reductase (GR) (type IV), and DTNB were obtained from Sigma. The enzymatic kinetics in the Eyer assay have been modified so that the GR catalyzed step is no longer rate limiting. Therefore, this method is much less susceptible to any endogenous intrasample GR inhibitors [26].

Rapid reaction kinetics were followed on a SLM-AMINCO 2C dual wavelength spectrophotometer, with the following instrument settings: 412 nm vs. 550 nm, 3 nm bandwidth, 0.05 Abs, 50 sec/in. Sample concentrations (nmol/ml) were determined by comparison with a 10-point standard curve conducted concurrently. The GSH standard curve ranged from 0.2 to 2 nmol/ml and was linear over its entire range. Results were expressed a nmol GSH/mg protein.

Evaluation of Cellular Peroxidase Activity

Because enzyme function may be altered after repeated subculturing, all catalase assays were conducted on cells that had undergone similar

low (≤ 10) passages in culture. Cells were harvested by trypsinization, the trypsin was stopped by a 20-fold excess of ice-cold PBS-0.02% EDTA, and cells centrifuged at 4°C, 1,500 rpm. Following three washes (samples resuspended and recentrifuged with each wash) with chilled PBS–0.02% EDTA, the cellular pellet was resuspended in PBS–0.02% EDTA, and a portion removed for protein determination. The remaining cells were centrifuged at 4°C and resuspended in 600 μl of 50 mM potassium phosphate buffer, containing 1% hydrogenated Triton (#648464, Calbiochem, La Jolla, CA). Cellular disruption was conducted by placing the cells in a sealed microfuge tube which was secured in an ice water bath subjacent to the sonication tip. Cellular samples were sonicated (Vibra Cell, Sonics and Materials Inc., Danbury, CT) for three 10-sec intervals (settings: pulsatile, 90% duty cycle, output control = 10), with a 10-sec interval between pulses, and a cellular aliquot obtained to assess adequacy of cellular disruption. If necessary, the sonication procedure was repeated. Cellular samples were microfuged at 14,000 rpm for 2 min and the supernate carefully decanted, placed on ice, and assayed within 2 h.

Cellular levels of peroxidase activity were determined in accordance with the method of Clairborne, using conditions specific for catalase [Claiborne, 1985]. The assay was conducted by evaluating the degradation of 10.3 mM H_2O_2 at pH 7.0, 25°C, 240 nm, on a Beckman DU 7400 spectrophotometer that contained a Kinetics/Time software package, and a Peltier Temperature Monitoring system. To account for any increased absorbance due to released cellular DNA, the absorbance at 260 nm was subtracted from the both the standard curve (for control purposes) and cellular samples. A catalase (Sigma, #C 3155, bovine liver) standard curve (0.1, 0.5, 1, 5, 10, units catalase/ml, with catalase standards prepared in the 1% Triton–50 mM potassium phosphate buffer) was conducted with each group of cellular catalase assays. One unit of catalase activity was defined as the amount of enzyme that degrades 1 μM of H_2O_2/min, while the [H_2O_2] decreases from 10.3 to 9.2 mM [Claiborne, 1985]. Cellular catalase activity was expressed as units catalase activity/mg protein. A >95% loss of enzymatic activity was demonstrated after sample incubation with 10 mM sodium azide for 30 min at 37°C.

Induction of Cellular Catalase Activity by Exposure to H_2O_2

To determine whether cellular basal levels of catalase activity were inducible by substrate exposure, cultures of HMECs and KS cells, grown in control medium, were exposed to exogenous H_2O_2.

Cultures of HMECs and KS cells were harvested, the cells replated at a density of 1.5×10^5 cells/ml in a M-199 base medium supplemented with 15 mM HEPES, *l*-glutamine (2.9 μg/ml), Na pyruvate (1.1 μg/ml), and 2% heat-inactivated FBS, and the cultures incubated for twelve hours at 37°C, 5% CO_2. Reduced serum levels were used to decrease overnight mitotic activity (retaining constant cell numbers) and to include some serum cellular attachment factors. The cultures were then gently rinsed with room temperature PBS, and the medium replaced with M-199 medium containing 15 mM HEPES, Na pyruvate, *l*-glutamine, and 20 μM H_2O_2. Cultures were incubated (37°C, 5% CO_2) for 60 min and then gently rinsed with room temperature PBS. Then, the complete control medium (*con*) (M-199 supplemented with 15 mM HEPES, sodium heparin (90 μg/ml), endothelial cell growth supplement (150 μg/ml), 15% heat-inactivated FBS, 5% heat-inactivated, pooled male human serum) was added and the cultures incubated for 24 h prior to harvest for determination of catalase activity. Photographs were taken, on a 37°C temperature-controlled stage, prior to, and immediately after, the addition of H_2O_2, and then at 5-, 15-, 30-, and 60-min intervals during the H_2O_2 exposure assay, and at the 24-h recovery point prior to culture harvest catalase evaluation.

RESULTS

To maximize KS cellular subpopulation recovery, all of the KS primary cultures were initiated in the viral product-rich KS milieu. Once the KS cultures were established in the KS milieu, some of the KS cells from all of the KS strains were subcultured in a control medium, (*con*) consisting of the same base medium, supplements, and serum, without the MOT-conditioned medium.

In many of the KS cultures, cellular growth characteristics were modified when KS cells were cultured in the control versus the KS milieu (Fig. 1). Cellular mitotic indices and growth rates were generally higher when cultures were grown in the KS milieu. Preliminary results

indicate that KS strains isolated from higher histologic grade KS lesions adapt more readily to culture in the control medium (Fig. 1). In control medium, the mitotic indices and growth rate of higher-grade KS strains mirrored growth in the KS milieu after a brief (48–72-h) cellular conditioning of the control medium (Fig. 1). Modifications in cellular growth characteristics were also observed during comparison of human microvascular endothelial cells (HMECs) cultured in either the control (*con*) or the KS milieu (*ksm*). HMECs cultured in *ksm* demonstrated a "growth relaxed" phenotype [Mallery et al., in press], lack of contact inhibition, anchorage independence, and failure to undergo differentiation/microtubule formation (Fig. 1).

Specific criteria have been employed to document that our cultures are KS in origin. First, a portion of all KS tissue explants was submitted for light microscopic examination to confirm the diagnosis of KS. Second, KS cells, during both control and KS milieu conditions, show growth characteristics consistent with a transformed phenotype-a lack of contact inhibition, loss of anchorage dependence, and a concurrent increase in intercellular adhesion. KS cells also possess a spindle cell morphology (Figs. 1–3), a recognized phenotypic feature of cultured KS cells [Corbeil et al., 1991; Miles et al., 1990; Roth et al., 1992]. Finally, KS cells demonstrated high autologous production of interleukin-6 (IL-6) [Miles et al., 1990]. When grown to approximately 80% confluency, washed, and cultured in serum-free medium for 48 h. KS cultures produced seven-fold higher levels of IL-6 in comparison to human microvascular endothelial cells (381.10 pg/ml vs. 56.73 pg/ml).

To determine the cellular bioenergetic status, HPLC nucleotide analyses were conducted on all KS and HMEC culture groups. Several uniform findings were found in the HPLC nucleotide profiles (Table I). As anticipated, due to its recognized position as the primary cellular energy reserve, ATP was the main high energy triphosphate in all culture groups. Furthermore, all culture groups possessed an energy charge (e.c. = [ATP] + 1/2[ADP]/[ATP] + [ADP] + [AMP]) indicative of ongoing, active oxidative metabolism (i.e., 0.96, 0.95, 0.96, and 0.97 for the KS-*con*, KS-*ksm*, HMEC-*con*, and HMEC-*ksm* cultures, respectively. In addition, the NAD^+/NADH redox pair was the primary form of nicotinamide nucleotides in all cultures.

In contrast to the uniform bioenergetic findings, other components of the HPLC nucleotide profiles demonstrated that there were also significant differences (Table I). These differences in nucleotide profiles reflected both the cell of origin and its culture conditions. During culture in the KS milieu, significant and opposite changes occurred in the HMEC and KS nucleotide bioenergetic profiles. Relative to all other culture groups, HMEC-*ksm* cultures possessed the highest, and KS-*ksm* cultures possessed the lowest, levels of high energy phosphates (ATP, GTP, UTP, CTP), total adenine nucleotides (ATP + ADP + AMP), and oxidized nicotinamide nucleotides (NAD^+). Relative to KS-*ksm* cells, KS-*con* cells contained significantly greater levels of ATP, total high energy phosphates (ATP + GTP + UTP + CTP), and NADPH. In contrast, during culture in the *ksm*, HMECs significantly increased their levels of high energy phosphates, total adenine nucleotides, NAD^+, and total nicotinamide nucleotides. Both of the KS culture groups possessed a highly reduced redox poise (ratio NAD(P)H/$NAD(P)^+$) (1.41 KS-*con*, 1.40 KS-*ksm*) in comparison to the redox states (0.90, 0.79) of the HMEC-*ksm* and HMEC-*con* cultures, respectively.

Cellular levels of GSH in cultured KS cells were independent of the culture medium, (Table II) and were significantly lower than GSH levels detected in either human microvascular endothelial cells or dermal fibroblasts. In contrast, human microvascular endothelial cell GSH levels reflected the culture milieu. When cultured in *ksm*, human microvascular endothelial cells possessed increased cellular levels of GSH. Levels of GSSG were nondetectable (< 0.01 nmol/ml) in all the culture groups.

Cellular levels of catalase activity reflected both the cell of origin and the culture conditions (Table II). Highest overall levels of catalase activity were demonstrated by dermal fibroblasts. While comparable levels of catalase activity were detected in HMECs and KS cells during culture in the control medium, significant and opposite cellular responses occurred during culture in *ksm*. HMEC-*ksm* cells significantly increased catalase activity to levels comparable to dermal fibroblasts. In contrast, KS-*ksm* cells showed a significant decrease in catalase relative to KS control cultures. Furthermore, during culture in *ksm*, there were significant differences between HMEC and KS catalase activities.

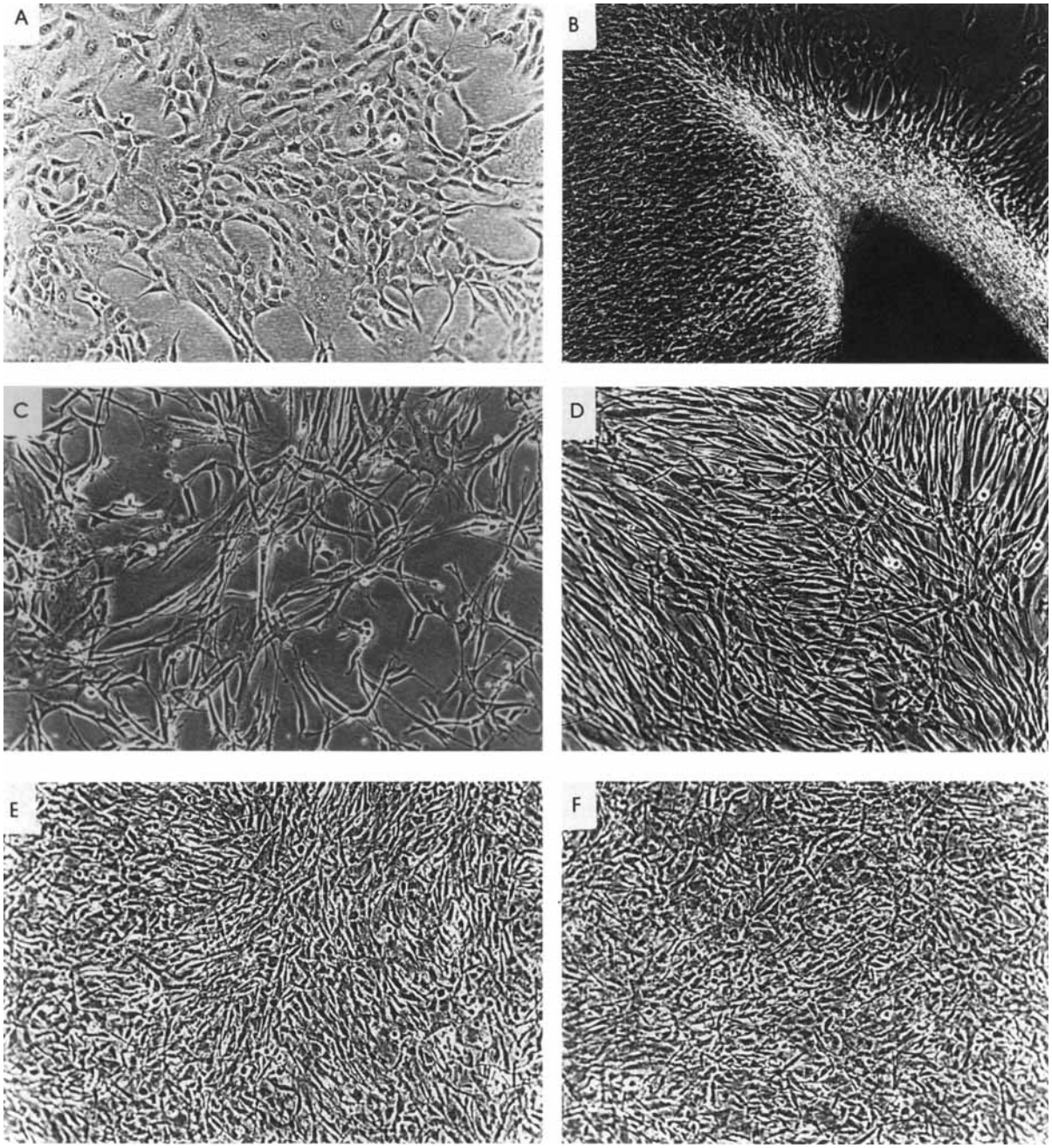

Fig. 1. Culture condition, and Kaposi's sarcoma (KS) histological grade, related modifications in cellular morphology and growth characteristics of human microvascular endothelial cells (HMECs), and AIDS-related KS cells during culture in either a control medium (*con*) (M-199 supplemented with 15 mM HEPES, 90 μg/ml sodium heparin, 150 μg/ml endothelial cell growth supplement, 15% heat-inactivated fetal bovine, 5% heat-inactivated, pooled, human male serum), or the "KS milieu" (*ksm*) (control medium supplemented by addition of 20% conditioned medium from MOT, an HTLV-II-infected cell line). Proliferative HMECs grown in *con* are characterized by a cobblestone morphology and anchorage dependence (**A**), while HMECs grown in *ksm* (**B**) show a loss of anchorage dependence, and formation of intercellular aggregates which detach from the culture flask. Cellular viability of the anchorage independent HMEC-*ksm* cellular aggregates was demonstrated by trituration and subsequent successful replating of detached HMECs. KS cells isolated from lower clinical stage (i.e., patch)/histological grade lesions demonstrated markedly slower growth rates and a more irregular phenotype during culture in the *con* (**C**) vs. the *ksm* (**D**) medium. In contrast, after a brief (24–48-h period) acclimation period, growth rates and cellular morphology of cells isolated from higher-grade KS lesions were similar in both the *con* (**E**) and *ksm* (**F**). All photomicrographs were taken at ×100.

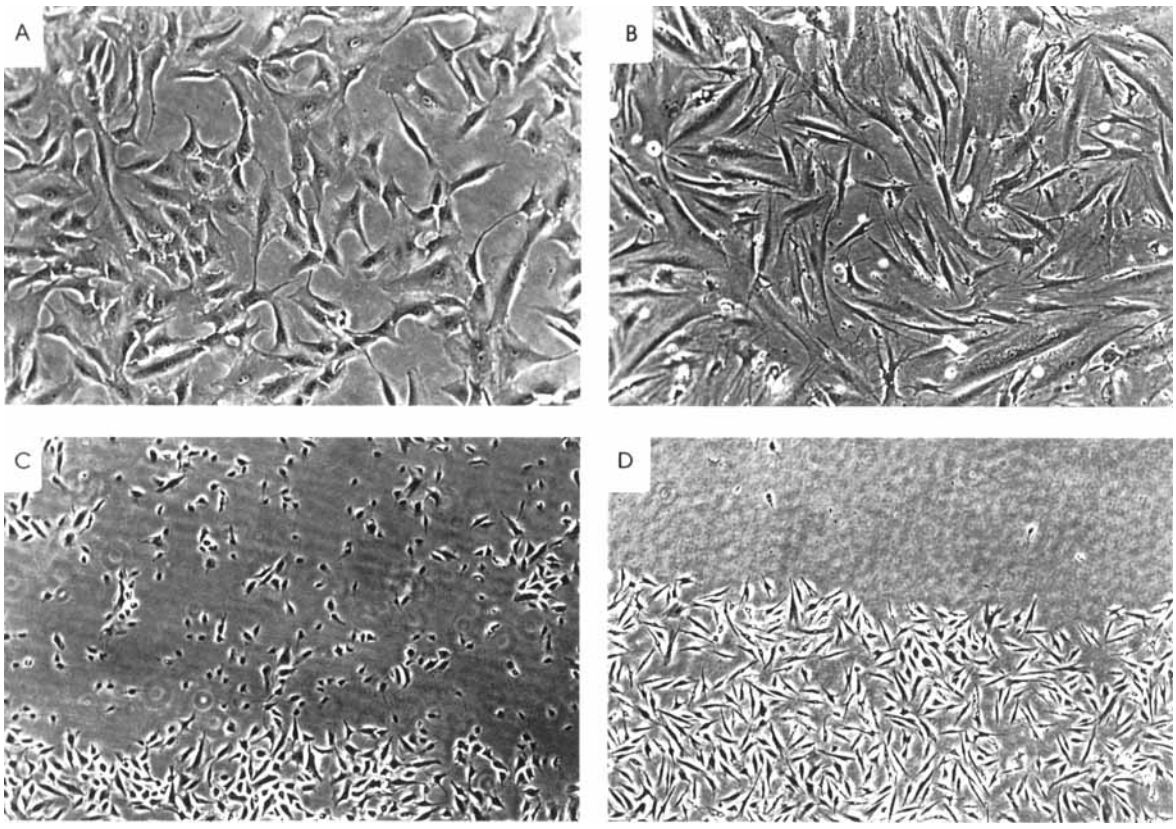

Fig. 2. Cultures of human microvascular endothelial cells (HMECs) and AIDS-related Kaposi's sarcoma (KS) cells were plated at a density of 1.5×10^5 cells/ml in a low serum medium (M-199 supplemented with 2% heat-inactivated fetal bovine serum, 15 mM HEPES, *l*-glutamine (2.9 μg/ml), Na pyruvate (1.1 μg/ml)), and incubated overnight at 37°C, 5% CO_2. Pretreatment photographs following overnight plating show that the reduced serum medium provided both good plating efficiency and reduction in mitotic activity (**A** = HMEC, **B** = KS, both ×100). The KS cellular strain depicted in these photographs produced birefringent spherical structures that, upon quick observation, may be mistaken for mitotic figures (B,D in both Figs. 2 and 3). The cultures were gently rinsed with room temperature phosphate-buffered saline, and the medium replaced with the M-199 base supplemented with *l*-glutamine, Na pyruvate, HEPES, which also contained 20 μM H_2O_2. Cellular response to the addition of H_2O_2 was very (within 2 min) rapid, and most pronounced at the peripheral, less densely packed, areas of the culture flasks. Cultures of HMECs (**C**) (×40) and KS (**D**) (×40) cells demonstrating marked evidence of cell lysis/destruction at the periphery of the flasks 5 min after the addition of H_2O_2.

Cellular response to exogenous H_2O_2 was dictated by both cell position in the flask and culture catalase activity. All the culture groups showed an increase in cellular density at the central portion of the flask, with peripheral cellular density remaining lower until culture confluence. The most severe cell damage/cell lysis occurred at the peripheral, less cell dense zones of the flask, while nonlethal perturbations (e.g., increases in intracytoplasmic granularity), were noted in the more densely packed, centrally located cells (Fig. 2). By 24 h, HMECs demonstrated "wound healing" behavior by migrating into, and mitotic activity at, zones of former cell destruction (Fig. 3). In contrast, at 24 h, KS cultures showed a lack of both migration and mitotic activity, as well as evidence of sustained cellular damage (e.g., numerous enlarged cells containing stress fibers) (Fig. 3).

Opposite cellular biochemical responses were observed in HMEC and KS cultures following exposure to exogenous H_2O_2 (Fig. 4). While the HMEC catalase activity showed a significant increase (46% increase above basal catalase activity, $\bar{x}$ units CAT ±SD/mg protein, 8.60 ± 6.34, n = 9, to 18.67 ± 6.47, n = 7), the KS cultures failed to positively respond and showed a 31% decrease in catalase (14.50 ± 4.85, n = 7, to 10.01 ± 4.87, n = 5). Furthermore, following H_2O_2 challenge, significant differences ($P < 0.05$) existed between HMEC and KS catalase activity (Fig. 4).

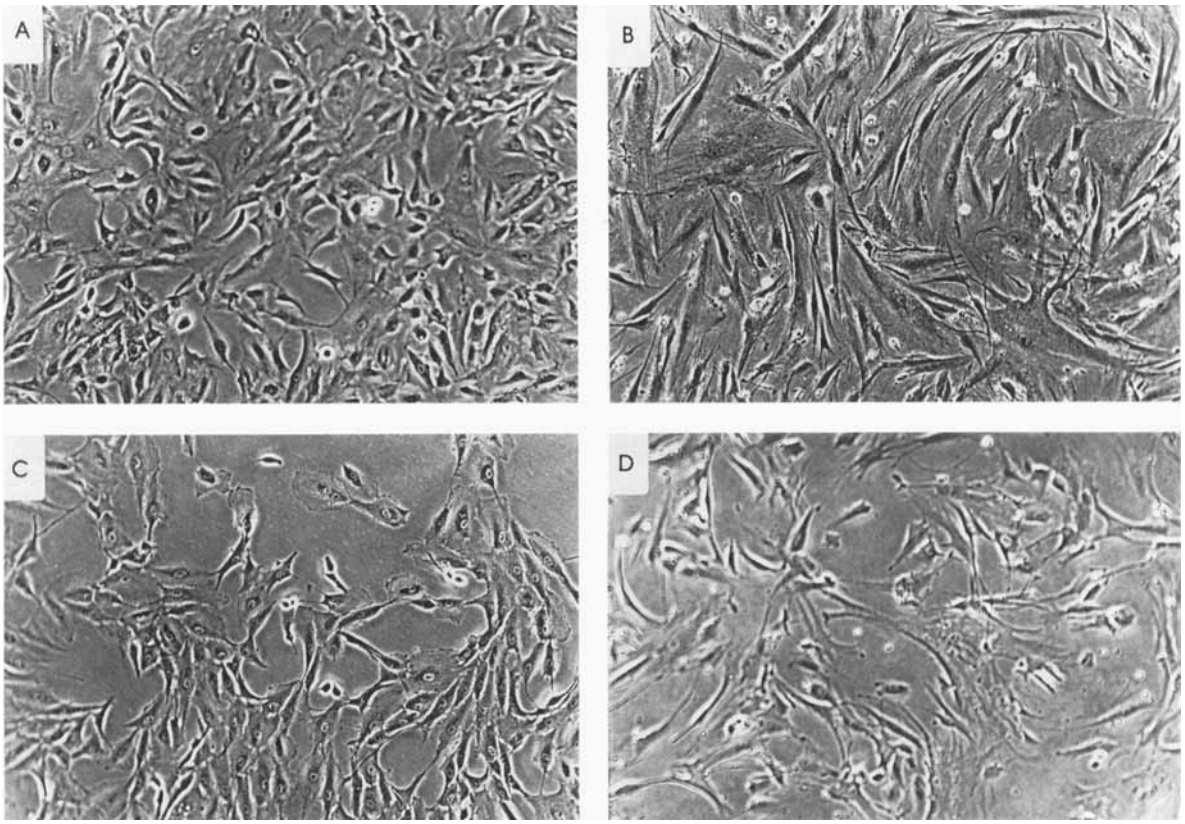

Fig. 3. Marked differences in culture related (human microvascular endothelial cell (HMEC) vs. AIDS-related Kaposi's sarcoma (KS)) adaptations to exposure to 20 μM exogenous H_2O_2. After a 24-h recovery in the complete control medium (M-199 supplemented with 15 mM HEPES, 90 μg/ml sodium heparin, 150 μg/ml endothelial cell growth supplement, 15% heat-inactivated fetal bovine serum, 5% heat-inactivated, pooled, human male serum) HMECs show mitotic activity, and cellular migration into the zones of former cell destruction (**A**) (×100), and (**C**) (×100). In contrast, KS cultures demonstrated an overall lack of positive adaptation following H_2O_2 challenge. At the 24-h recovery time point, KS cultures show both a lack of mitotic activity (**B** and **D**, KS culture photomics) (×100), and (**D**) (×100), in addition to markedly enlarged cells with evident stress fibers (D) (×100).

DISCUSSION

In this paper, we report data that begins to biochemically characterize AIDS-KS cells, and provides insight into how AIDS-KS may be initiated, and/or promoted by means of oxidant stress. Observation of KS growth characteristics showed that growth adaptation to culture in the control medium correlated with lesional clinical stage and histological grade. KS cultures that were isolated from KS lesions at an advanced clinical stage (multifocal, nodular) and grade adapted readily, as demonstrated by mitotic indices and growth rates, to control culture conditions. These findings are consistent with the observation that higher-grade lesional cells are frequently better autologous producers of growth factors than their lower-grade counterparts [Pitot, 1986]. Studies are currently ongoing to determine if KS culture production of specific KS growth-promoting cytokines (e.g., IL-6) correlates with lesional stage and grade.

Nucleotide analyses that we have previously conducted on HMECs cultured in a control medium demonstrated that as a component of their differentiated to proliferative growth state transition, HMECs modify their bioenergetic profiles [Mallery et al., 1993], and that there is a significant difference in the bioenergetic profiles of HMECs cultured in the KS milieu in comparison to the control medium [Mallery et al., 1994]. The current nucleotide profiles showed, as was also noted in our previous study, several uniform nucleotide findings, which were independent of cell type or culture conditions. In all the cellular cultures, as is true in most cells, ATP was the primary high-energy phosphate [Martin

TABLE I. HPLC Nucleotide Analysis/Bioenergetic Profiles of Cultured Human Microvascular and Kaposi's Sarcoma Cells†

Nucleotide	KS-*con*	KS-*ksm*	HMEC-*con*	HMEC-*ksm*
AMP	0.47 ± 0.20	0.51 ± 0.13	0.46 ± 0.26	0.50 ± 0.14
ADP	2.48 ± 0.83	2.40 ± 0.60	1.68 ± 0.16	2.19 ± 0.24
ATP	41.43 ± 9.70[g]	29.05 ± 4.09[g,j]	31.86 ± 4.68[a]	51.78 ± 10.32[a,j]
Total (AMP + ADP + ATP)	44.38[h]	31.96[h,j]	34.00[b]	54.47[b,j]
NAD^+	4.93 ± 1.67[l]	4.61 ± 0.77[j]	5.55 ± 1.27[b]	9.26 ± 2.40[b,j,l]
NADH	6.76 ± 2.26	6.66 ± 2.09	4.54 ± 1.04[b]	6.59 ± 0.94[b]
Total NAD^+ + NADH	11.69[n]	11.27[k]	10.09[b]	15.85[b,k,n]
$NADP^+$	0.60 ± 0.23	0.40 ± 0.10	0.29 ± 0.10	0.31 ± 0.14
NADPH	1.03 ± 0.17[f]	0.37 ± 0.18[d,f,j]	0.98 ± 0.19[d]	0.96 ± 0.14[j]
Total $NADP^+$ + NADPH	1.63[f]	0.77[e,f,k]	1.27[e]	1.27[k]
GTP	5.44 ± 2.11[m]	4.92 ± 0.23[j]	5.74 ± 0.91[a]	8.08 ± 0.91[a,j,m]
UTP + CTP	3.40 ± 1.66	2.34 ± 0.41[k]	3.03 ± 0.47[c]	4.21 ± 1.13[c,k]
Total ATP + GTP + UTP + CTP	50.27[i]	36.31[i,j]	40.63[b]	64.07[b,j]

†HPLC nucleotide analyses of human Kaposi's sarcoma cells (KS) and human microvascular endothelial cells (HMECs) assayed during culture in either the control medium (*con*) (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% pooled, human male serum), or the KS milieu (*ksm*) (control medium supplemented by addition of 20% conditioned medium from MOT, an HTLV II infected cell line). Results expressed as mean nmol/mg protein ±SD, n = 7 for the KS-*con* cultures, n = 9 for the KS-*ksm* cultures, n = 10 for the HMEC-*con* cultures, n = 11 for the HMEC-*ksm* cultures. Statistical analyses were conducted by the Kruskal–Wallis one-way analysis of variance, followed by the Mann–Whitney U (two-tailed) test. HMEC-*con* vs. HMEC-*ksm:* a = $P < 0.001$, b = $P < 0.002$, c = $P < 0.02$, HMEC-*con* vs. KS-*ksm:* d = $P < 0.001$, e = $P < 0.002$, KS-*con* vs. KS-*ksm:* f = $P < 0.002$, g = $P < 0.02$, h = $P < 0.04$, i = $P < 0.05$, HMEC-*ksm* vs. KS-*ksm:* j = $P < 0.001$, k = $P < 0.002$, HMEC-*ksm* vs. KS-*con:* l = $P < 0.002$, m = $P < 0.02$, n = $P < 0.05$.

TABLE II. Cellular Cytoprotective Capabilities of Cultured Human Fibroblasts Microvascular Endothelial and Kaposi's Sarcoma Cells†

Cell type	Culture conditions	[GSH]/mg protein	Units CAT/mg protein
KS	KS milieu	10.42 ± 5.75, n = 13[b,d,f]	9.32 ± 4.85, n = 10[i,j,k]
KS	Control medium	9.99 ± 4.13, n = 9[a,c,e]	14.50 ± 4.92, n = 9[k]
HMECs	KS milieu	64.57 ± 23.15, n = 7[c,d]	21.22 ± 11.47, n = 7[g,j]
HMECs	Control medium	46.38 ± 10.47, n = 9[a,b]	8.60 ± 6.34, n = 9[g,h]
Dermal fibroblasts	Enriched EMEM	39.75 ± 8.53, n = 8[e,f]	21.40 ± 11.91, n = 9[h,i]

†Cellular cytoprotective capabilities, as assessed by determination of levels of glutathione (GSH), and units of catalase activity (CAT), following culture harvest during proliferative growth conditions. Human microvascular endothelial cells (HMECs), and human Kaposi's sarcoma cells (KS) were cultured in either a control medium (*con*) (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), or the "KS milieu"—(*ksm*) control medium supplemented by addition of 20% conditioned medium from MOT, an HTLV-II-infected cell line). Dermal fibroblasts were cultured in Eagle minimum essential medium supplemented by addition of 1.5× essential amino acids, 1.5× vitamins, 2× nonessential amino acids, 1× *l*-glutamine, and 10% heat inactivated fetal bovine serum (enriched EMEM). Results are expressed as mean levels (nmol for GSH, units of activity for CAT) per mg of protein ±SD. Statistical analyses were conducted by the Kruskal–Wallis one way analysis of variance followed by the Mann-Whitney U (two-tailed) test. GSH statistics: a = $P < 0.001$ HMEC-*con* vs. KS-*con*, b = $P < 0.001$ HMEC-*con* vs. KS-*ksm*, c = $P < 0.001$ HMEC-*ksm* vs. KS-*con*, d = $P < 0.001$ HMEC-*ksm* vs. KS-*ksm*, e = $P < 0.001$ fibroblasts vs. KS-*con*, f = $P < 0.001$ fibroblasts vs. KS-*ksm*. Catalase statistics: g = $P < 0.05$ HMEC-*con* vs. HMEC-*ksm*, h = $P < 0.02$ HMEC-*con* vs. fibroblasts, i = $P < 0.002$ KS-*ksm* vs. fibroblasts, j = $P < 0.02$ HMEC-*ksm* vs. KS-*ksm*, k = $P < 0.002$ KS-*con* vs. KS-*ksm*.

et al., 1985; Matthews and van Holde, 1990]. Furthermore, all culture groups possessed energy charges ≥ 0.95. These high-energy levels demonstrate that during culture both HMECs and KS cells maintain high rates of oxidative metabolism. Another consistent finding in the bioenergetic profiles was that NADH/NAD^+ was the primary redox pair and that NADH was the primary form of reduced nicotinamide nucleotides. These results are in agreement with nicotinamide nucleotide distributions in other cells, and likely reflect ongoing activity of the NAD

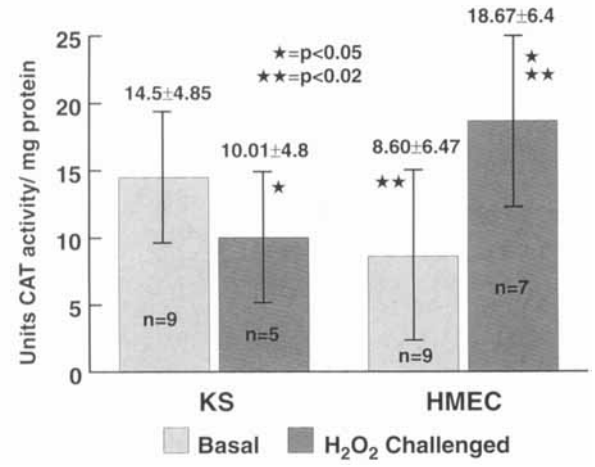

Fig. 4. Comparison of basal and H_2O_2 stimulated levels of catalase (CAT) activity detected in cultured human microvascular endothelial cells (HMECs) and AIDS-related Kaposi's sarcoma (KS) cells. Basal CAT levels were obtained from proliferative cultures, passage <10, during culture in control medium (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 (FBS), 5% heat-inactivated, pooled, human male serum). For the H_2O_2 challenge, cells were plated at a density of 1.5×10^5 cells/ml in a M-199 base medium supplemented with 15 mM HEPES, *l*-glutamine (2.9 μg/ml), Na pyruvate (1.1 μg/ml), 2% heat inactivated FBS, and incubated at 37°C, 5% CO_2, overnight. The cultures were then gently rinsed with room temperature phosphate-buffered saline (PBS), and the low serum medium replaced with a M-199 base medium (M-199 supplemented with HEPES, *l*-glutamine, and Na pyruvate) that contained 20 μM H_2O_2. The H_2O_2 challenged cultures were incubated for 1 h, 37°C, 5% CO_2. The cultures were then gently rinsed with room temperature PBS, control medium added, and cultures incubated for 24 h prior to harvest for determination of CAT activity. Statistical analyses were conducted by the Kruskal Wallis one-way analysis of variance, followed by the Mann-Whitney U (two-tailed) test.

linked dehydrogenases used in the oxidative metabolic pathways [Geisbuhler et al., 1984; Mallery et al., 1993; Martin et al., 1985; Matthews and van Holde, 1990].

Although similarities were found in the nucleotide profiles, there were also differences that reflected both the culture conditions and cell of origin. When KS cells were cultured in the KS milieu, they still maintained a high-energy charge but contained significantly lower levels of ATP and had apparently become more glycolytic [Pitot, 1986]. This observation, whereby a neoplastic cell becomes more reliant on anaerobic pathways, is consistent with the biochemistry of many malignant cells [Pitot, 1986]. Therefore, KS cell culture in the virus-induced cytokine-rich KS milieu may allow a more representative bioenergetic profile of KS cells to emerge. Significantly lower cellular NADPH levels were another nucleotide difference noted during comparison of KS *ksm* cultures with either KS *con* or either HMEC culture group. This finding likely reflects a decrease in activity of NADPH-generating enzymes/pathways (e.g., malic enzyme, hexose monophosphate shunt) in KS-*ksm* cultures [Martin et al., 1985; Matthews and van Holde, 1990]. Because NADPH provides the reducing equivalents for an enzyme used for cytoprotection (GSSG reductase), the cytokine-rich KS milieu decreases KS capacity to cope with oxidant stress [Halliwell and Aruoma, 1991; Meister, 1988].

However, the possession of reducing equivalents per se was not a problem in either KS culture groups, as both the control and KS milieu cells possessed a highly reduced redox poise. It is improbable that these redox states reflect cellular hypoxia or a culture-handling artifact. First, all cells were harvested under aerobic conditions. Also, during hypoxia, cellular ATP levels would plummet as cells shift to anaerobic metabolism [Martin et al., 1985; Matthews and van Holde, 1990]. However, the KS cultures' energy charges demonstrate that cellular energy status was not perturbed during harvesting for HPLC analysis. Therefore, our findings suggest that a high degree of dehydrogenase activity is maintained in cultured KS cells [Martin et al., 1985; Matthews and van Holde, 1990].

Relative to either of the KS culture groups or the HMEC-*con* cultures, HMEC-*ksm* contained the highest total levels of adenine nucleotides, nicotinamide nucleotides, and high-energy phosphates. These finding demonstrate increased rates of nucleotide synthesis and turnover in the HMEC-*ksm* cultures [Martin et al., 1985; Matthews and van Holde, 1990] and are in agreement with our earlier observations that, when cultured in the KS milieu, HMECs assume a highly proliferative "growth relaxed" phenotype [Mallery et al., 1994].

The levels of GSH detected in KS cultures were both independent of the culture conditions and significantly lower than the GSH levels determined in either HMECs or fibroblasts or GSH fibroblast levels reported by other investigators [Mallery et al., 1991; Mbemba et al., 1985]. A reduction in GSH levels would impair key GSH-dependent cytoprotective enzymes (i.e., GSH-S-transferases and GSH peroxidase) [Meister, 1988], resulting in a decreased KS cellular capacity to either detoxify xenobiotics or inactivate

organic peroxides. In contrast to the KS cultures, HMEC GSH levels did reflect culture conditions. HMECs adapted to the KS milieu by increasing their GSH levels. Previously, we have reported that HMECs show phenotypic and functional modifications (e.g., failure to differentiate), when cultured in the KS milieu [Mallery et al., 1994]. This increase in GSH content not only aids in cytoprotection but facilitates cellular retention of a proliferative phenotype. The additional reducing equivalents provided by GSH (both as GSH and glutaredoxin) help maintain the activity of two thiol-dependent enzymes used during DNA synthesis—DNA polymerase α and ribonucleotide reductase [Matthews and van Holde, 1990; Meister, 1988].

Basal levels of catalase activity were determined in both the control and KS milieu culture groups, as well as dermal fibroblasts. Fibroblasts were included in the catalase assays to provide both an internal and external basis for comparison. While human fibroblast catalase levels have been reported [Yohn et al., 1991], there are no comparable reported catalase levels for either HMECs or KS cells. Furthermore, because KS cells are maintained to be of mesenchymal origin, the inclusion of fibroblasts in addition to HMECs, provides two mesodermally derived populations for KS catalase activity comparison.

Our levels of fibroblast catalase activity ($\bar{x}$ = 21.4 Units/mg protein) are slightly increased relative to levels reported by Yohn et al. ($\bar{x}$ = 12.5 U/mg) [Yohn et al., 1991]. Because humans are an outbred population, these variations in catalase levels may reflect differences in donor enzyme function, as well as differences in culture methodology such as number of passages and enzyme preservation during cellular harvest.

While comparable levels of catalase activity were found between KS and HMECs during control culture conditions, significant and opposite catalase activity responses were detected during KS milieu growth. The increase in HMEC catalase function in the presence of MOT conditioned medium reflects a nonspecific HMEC enzyme stimulation during culture in the KS milieu. Possibly, the conditioned medium contains MOT metabolic byproducts that contain, or resemble, peroxides. It was these differences in catalase activity, triggered by a rather nonspecific stimulus, that prompted the H_2O_2 exposure studies. Due to the significantly higher HMEC basal catalase activities detected during culture in the KS milieu, HMEC and KS cultures used for the induction of catalase were grown in the control medium to avoid an HMEC bias.

There were several reasons that we elected to use a 20-μM concentration of H_2O_2 during the catalase induction assays. Because we wanted to stimulate catalase, and not glutathione peroxidase, we selected a concentration of H_2O_2 that was sufficiently high to be preferentially metabolized by catalase [Martin et al., 1985; Matthews and van Holde, 1990; Sandstrom and Marklund, 1990], but not so great as to kill the majority of exposed cells [Schraufstatter et al., 1985, 1988; Jonas et al., 1989]. Finally, a 20-μM concentration of H_2O_2 is well within the physiological range that could be achieved by an activated phagocyte [Fantone and Ward, 1982; Halliwell and Aruoma, 1991; O'Donnell-Tormey et al., 1985; Roos et al., 1980].

Following exposure to H_2O_2, there is an oxidation of both the cellular redox poise and GSH status, depletion of ATP levels, and an activation of an enzyme necessary for DNA repair—poly(ADP-ribose)polymerase [Sandstrom and Marklund, 1990; Schraufstatter et al., 1985, 1988; Spragg et al., 1985; Hyslop et al., 1988]. Cellular replenishment of NADPH by the hexose monophosphate shunt is contingent upon glucose availability [Martin et al., 1985; Matthews and van Holde, 1990], while the source of ribose moieties used by poly(ADP-ribose)polymerase is NAD^+ and the NAD precursors ATP and glutamine [Matthews and van Holde, 1990]. Our conditions employed during H_2O_2 exposure (basal medium supplemented with HEPES, *l*-glutamine, and Na pyruvate) provided the challenged cells with the necessary components (e.g., carbon sources) to respond to the H_2O_2-induced oxidation.

There were two reasons that a 24-h interval was included between H_2O_2 challenge and determination of cellular catalase activity. First, this recovery time was included to afford responsive cells the chance to replenish their thiol redox bioenergetic status. Second, detection of increased catalase activity 24 h after H_2O_2 exposure would demonstrate cellular capacity not only to transiently up-regulate, but also to sustain, levels of an integral cytoprotective enzyme. Because AIDS patients frequently experience prolonged inflammatory states [Miles, 1992; Tappero et al., 1993], resulting in multiple episodes of ROS generation, cellular biochemical sus-

tained responsiveness is essential to prevent cellular damage from future oxidant stress.

Both the HMEC and KS cultures showed increased cell destruction at the peripheral portion of the flasks during H_2O_2 exposure. This finding is consistent with the observed central (increased) vs. peripheral (decreased) differences in cellular plating densities and likely reflect a cell number associated cumulative effect on local catalase activity. However, following H_2O_2 exposure, only the HMEC cultures demonstrated morphological evidence of positive cellular adaptation. Within 24 h after H_2O_2 challenge, HMECs showed "wound healing" via migration into, and mitotic activity at, zones of former cell destruction. These HMEC-KS cellular adaptative differences likely reflect the better intrinsic (increased GSH, NADPH) cytoprotective capacity of HMECs. Studies are ongoing to characterize the HMEC and KS thiol redox bioenergetic responses during, and following, H_2O_2 challenge.

Following H_2O_2 exposure, only the HMECs responded with an induction of catalase activity. There are two routes by which the HMECs could have upregulated catalase activity: (1) by increasing the absolute concentration of enzyme by either decreasing catalase degradation and/or increasing catalase synthesis [Martin et al., 1985; Matthews and van Holde 1990] and (2) by increasing the catalytic efficiency of catalase [Martin et al., 1985; Matthews and van Holde, 1990]. Because metal ions are a frequent modulator of enzyme catalytic efficiency [Martin et al., 1985], the hemoprotein, catalase, may be regulated by both routes.

While cultured KS cells demonstrated basal catalase expression, they failed to increase enzymatic activity after H_2O_2 exposure. Because substrate induction of enzymatic activity is genetically determined [Martin et al., 1985], our results imply that AIDS-KS cells have an inherent inability to respond to levels of H_2O_2 found at inflammatory sites. This failure of KS cell catalase induction, in conjunction with their significantly lower GSH levels, and their reduction in both NADPH and catalase during culture in the KS milieu, demonstrate a marked deficit in cytoprotection in KS cells. In conclusion, our results imploy that oxidant stress is implicated in the development of AIDS-related KS, and that local intervention with antioxidant therapy may prove beneficial in preventing disease progression.

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