EXPRESSION OF INTERLEUKIN-6 RECEPTORS AND NF-κB IN AIDS-RELATED KAPOSI SARCOMA CELL STRAINS

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ABSTRACT

AIDS-related Kaposi sarcoma (AIDS-KS) is the most common malignancy associated with HIV infection, with an incidence of 10-30% of all AIDS patients. As such, there have been a large number of AIDS-KS cell strains isolated and numerous studies conducted to elucidate the mechanisms of malignancy in this disease. We have reported histological grade associated differences in the ability of AIDS-KS cell strains to proliferate under conditions of minimal growth factor supple-mentation, with strains derived from high grade lesions having enhanced proliferation potential. Furthermore, we found that this difference in in vitro *growth characteristics was not attributed to grade associated differences in autologous growth factor release. These current investigations explored the hypothesis that grade associated growth differences could be attributed to differences in the expression of the components of the IL-6 receptor, or expression/inducibility of the pleotrophic transcription factor NF-κB. We determined there were no significant grade associated differences in the expression of either component (IL-6R α chain or gpl30) of the IL-6 receptor. However, non-lesional oral derived cell strain lysates from AIDS-KS patients (n=4) contained significantly lower concentrations of both components of the IL-6 receptor than AIDS-KS strains (n=8) and lower concentrations of gp-130 than normal human oral derived fibroblasts (n=2).*

Comparative analysis of sera concentrations of soluble components of the IL-6 receptor did not demonstrate significant differences between HIV+/KS+(n=7), HIV+/KS-(n=9) and normal (HIV-/KS-) (n=4) populations. Further, no differences were detected in the expression of NF-κB in AIDS-KS cell strains (n=5) derived from both high and low histological grade lesions as compared to non-lesional AIDS-KS cell stain (n= 1) and normal human oral derived fibroblasts (n=2) under conditions of: constitutive/proliferative growth, sera starvation, oxidative stress, and mitogen reintroduction after sera starvation. In conclusion, these investigations have eliminated two explanations for histological grade associated differences for in vitro *growth potential of AIDS-related KS cell strains and further substantiated the lack of systemic paracrine cytokine/cytokine receptor effects in AIDS-KS pathogenesis.*

AIDS-related Kaposi sarcoma (AIDS-KS) is the most common malignancy associated with HIV infection, with an overall incidence of 10-30% of all AIDS patients (1,2). Incidence rates are as high as 50% in the homosexual male sub-population of AIDS patients (3), suggesting possible additional risk factors among this risk group. Due to the high incidence of AIDS-KS, there have been a large number of *in vitro* cell culture models developed to determine the biological charac-teristics of these lesional cells. The vast majority of AIDS-KS cell strains have been

derived from epidermal (4-8) or lung biopsies and/or peritoneal/pleural effusion material (4,8-14), with only limited strains of oral mucosal origin (8,15-16).

Analysis of AIDS-KS cell strains have centered around the hypothesis that lesional cells produce autologous growth factors that allow for sustained grown potential both *in vitro* and *in vivo* (17,18). These investigations have revealed a large spectrum of cytokines that are autologously produced by AIDS-KS cells: (Interleukin) IL-1α, IL-1ß, IL-2, IL-6, (tumor necrosis factor)TNF-α, TNF-ß, (fibroblast growth factor) FGF-1, FGF-2, FGF-5, FGF-6, epidermal growth factor, Oncostatin M (OSM), (interferon) IFN-α, IFN-γ, (granulocyte macrophage-cloning stimulating factor) GM-CSF, TGF-α, TGF-ß, and (platelet derived growth factor) PDGF (4,7,10,11,13,19-33). Interpretation of these studies is difficult due to the variabilities in AIDS-KS cell strains utilized by different investigators, and variable methods of analyses. Furthermore, all *in vitro* analyses of AIDS-KS strains are hampered by the lack of a definitive AIDS-KS associated cell marker, which would assure selective analysis of only lesional AIDS-KS cells (17,18). In spite of the concentrated research focus, there have been limited conclusions as to the role of cytokines in the initiation and/or progression of AIDS-KS *in vivo*. However, one common characteristic of AIDS-KS cell strains is their capacity to release high concentrations of IL-6 (7,10,11,21,33). In order for IL-6 to function as an autologous growth factor, both components of the IL-6 receptor [IL-6R (α chain), and gp-130 (ß chain)] have to be present (34). Analysis of the expression of IL-6 receptor components in AIDS-KS strains demonstrate conflicting results with reports of their presence (10,35) and absence (8).

One mechanism which may account for the unregulated expression of cytokines by AIDS-KS cell strains is via the transcription factor NF-κB. NF-κB is a transcription factor with the potential to transactivate a wide variety of genes due to the abundance of homologous binding sites in various genes, inclusive of cytokine genes (36,37). Under unstimulated conditions NF-κB is located in the cytoplasm in an inactive form complexed to an inhibitory protein IκB. Cellular activation (via stress, mitogens) results in phosphorylation and rapid proteolysis of the IκB component, resulting in nuclear translocation of NF-κB and subsequent binding to the promoter regions of genes with homologous binding sites and stimulating gene activation (36). Expression of NF-κB in AIDS-KS strains is of interest for several reasons: 1. the association of NF-κB with the HIV *tat* gene product, which is a mitogen for AIDS-KS strains (22,28,38-40), 2. association of altered NF-κB control in other malignancies and/or disease states (41-46), 3. association of NF-κB with oxidative stress (47-50), a theoretical mechanism of AIDS/KS initiation and/or promotion (15,16), 4. ability of NF-κB to activate cytokines, such as IL-6, which are produced by AIDS-KS strains (36,37,51).

Our laboratory has derived AIDS-KS cell strains from lesional biopsies that were histologically evaluated as either high-grade (oral) or low-grade (epidermal). Notably, the majority of oral mucosal AIDS-KS lesions were high histological grade, while epidermal lesions were generally low grade lesions. In addition, our laboratory has derived control cell strains inclusive of nonlesional oral tissue from AIDS-KS patients (n=4) and oral tissue from normal (HIV-) individuals. Analysis of the AIDS-KS cell strains allowed us to determine that the cell strains derived from high-grade (oral) lesions have the ability to proliferate long term in medium with minimal supplementation of growth factors, in contrast to AIDS-KS strains from low-grade (epidermal) lesions (52). We hypothesized that the histological grade associated differences in growth characteristics were attributable to differences in constitutive cytokine production. However, we found that there were no histological grade associated differences in the constitutive expression of

cytokines: IL-1ß, IL-4, IL-6, TNF-α, TNF-ß, GM-CSF, OSM, PDGF, and FGF-2 (33). In these current investigations, we explore two additional hypotheses for the grade-associated differences in AIDS-KS cell strains: The increased growth potential of strains from high grade lesions reflects increased constitutive expression of: 1. IL-6 receptors, and/or 2. NF-κB. We report here that all AIDS-KS derived cell strains (regardless of histological grade of the primary AIDS-KS lesion) express significantly increased concentrations of IL-OR (α chain) relative to AIDS-KS non-lesional cells. In addition, there is no difference in the constitutive or inducibility of the NF-κB transcription factor in AIDS-KS cell strains when compared with AIDS-KS non-lesional cells or normal (HIV-) fibroblasts.

MATERIALS AND METHODS

Cell Strains and Growth Conditions

The cell strains for these investigations were, as previously described (15), biopsy-confirmed AIDS-KS cell strains from lesions of both high (n=5) and low (n=3) histological grade. In addition, we derived cell strains from oral-mucosal fibroblasts (n=2) and non-lesional AIDS-KS patient oral mucosal fibroblasts (n=4). All cells were maintained in EC media which consists of: Medium 199 (Life Technologies, Gaithersburgh, MD), 2.9 µg/ml of L-glutamine, 1.1 µg/ml of sodium pyruvate, 15 mM Hepes, 90 µg/ml sodium heparin, 120 µg/ml of endothelial cell growth supplement (prepared from bovine brain), 15 % heat-inactivated fetal bovine sera, and 5 % heat-inactivated human male sera. Cells were maintained in flasks coated with human fibronectin.

IL-6 Receptor Analysis

Cells were harvested at passage 5-7, via trypsinization, at a cell density of approximately 80% confluency. Culture supernates were aliquoted and stored at -70°C until analysis. Cell pellets were counted, washed 3x with phosphate buffered saline (PBS), and resuspended in PBS at a density of 2×10^8 cell/ml, and mixed with an equal volume of lysis buffer [1% NP-40, l mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF) in PBS], to a final cell density of 1×10^8/ml. The cell solution was incubated on ice from one hour with frequent vortexing. Cell lysate was stored at -70°C until analysis.

Fresh sera was obtained from a population of HIV+/KS+ (n=7), HIV+/KS- (n=9), and normal HIV- (n=4) individuals. Sera was aliquoted and stored at -70°C until analysis.

Concentrations of IL-6R (α-chain) and gp-130 (ß-chain) were determined as per manufacturer's instructions using duplicate wells for each sample in ELISA kits for soluble-IL-6R and gp-130 (R & D Systems, Minneapolis, MN) .

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was carried out as described (53) with the following modifications: cells that were washed twice with PBS were resuspended in 2 packed cell volumes of Buffer A [10 mM Hepes, pH 8, 10 mM KCl, 2 mM $MgCl_2$, 200 mM sucrose, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mg/ml antipain, 1µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 % Nonidet P-40]. Cells were incubated on ice for 15 minutes, vortexed and pelleted for 1 minute at 14 k x *g*. The resulting pellet was rinsed once with buffer A and resuspended in 2/3 volume of Buffer B [50 mM Hepes, pH 8, 2 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM Dithiothreitol, 0.5 mM PMSF, 1 mg/ml antipain,1 µg/ml aprotinin, 1 µg/ml leupeptin]. Cells were mixed gently for 30 minutes at 4°C on a rocker, then quick freeze-thawed 3x with liquid nitrogen, followed by pelleting debris for 5 minutes at 14 k x *g*. The supernate was diluted 1:1 with Buffer C [50 mM Hepes, pH 8, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mg/ml antipain, 1 µg/ml aprotinin,
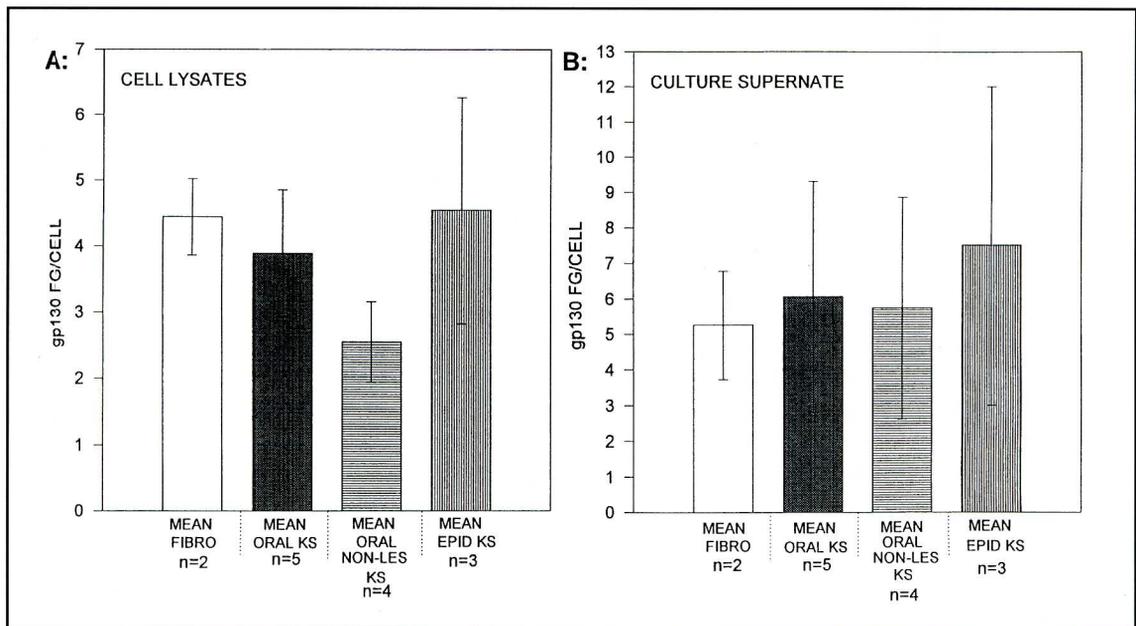
Fig. 1. *ELISA quantitation of gp-130 concentrations: Cells and supernates were harvested at 80 % confluence of culture at passage 5-7. Duplicate wells were averaged for each sample and mean values reported for 4 groups: Normal HIV-/KS- oral-derived fibroblasts (n=2)(FIBRO), Oral-derived AIDS-KS cell strains (n=5)(ORAL KS), Oral-derived non-lesional cell strains from HIV+/KS+ patients (n=4)(ORAL NON-LES KS), Epidermal derived AIDS-KS cell stains (n=3)(EPID KS). A: Cell lysates from harvested cells at a density of $1x10^8$ cell/ml. Statistical analysis (Kruskal-Wallis/MannWhitney) detected significant inter-group significant differences: Fibro vs Non-lesional KS p=0.004, Oral-derived KS vs Non-lesional KS p=0.004, Epidermal derived KS vs Non-lesional KS p=0.01. B: Analysis of culture supernates. There were no statistically significant inter-group differences.*

1 µg/ml leupeptin, 20% glycerol] and stored at -70°C until assay. Protein concentrations of nuclear extracts were determined with the Lowry protein assay using bovine gammaglobulins as the protein standard (54).

Fifteen µg of nuclear proteins were mixed with or without a fifty-fold (3.5 pmole) excess of competitive un-labeled oligonucleotides in 1x EMSA binding buffer: 20% glycerol, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl, 50 µg/ml Poly (dI-dC)•Poly (dI-dC) (Pharmacia, Piscataway, NJ) [total reaction volume 28 µl] and allowed to incubate at room temperature for 10 minutes. γ-P^{32} ATP (3000 Ci/mmole, 10 mCi/ml) (ICN Pharmaceuticals, Irvine, CA) end labeled consensus oligonucleotides were added (0.07 pmole) to the reaction and allowed an additional 20 minutes incubation at room temperature. The reaction was stopped by the addition of 10 x loading buffer (250 mM Tris-HCI pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, 40% glycerol), followed by separation of samples in a 4% nondenaturing polyacrylamide gel in 0.5 x TBE running buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA). Gels were run until the dye front ran off the bottom of the gel. Gels were dried and exposed to X-ray film for development. Positive controls for each run included use of HeLa nuclear extract (Promega, Madison, WI), with Ig-κB target oligonucleotides, and negative control of reagents without any nuclear extracts. Consensus oligonucleotide sequences were: (5' to 3'), double stranded: Ig-κB AGT TGA GGG GAC TTT CCC AGG C, (Promega, Madison, WI) IL-6-κB AAA TGT GGG ATT
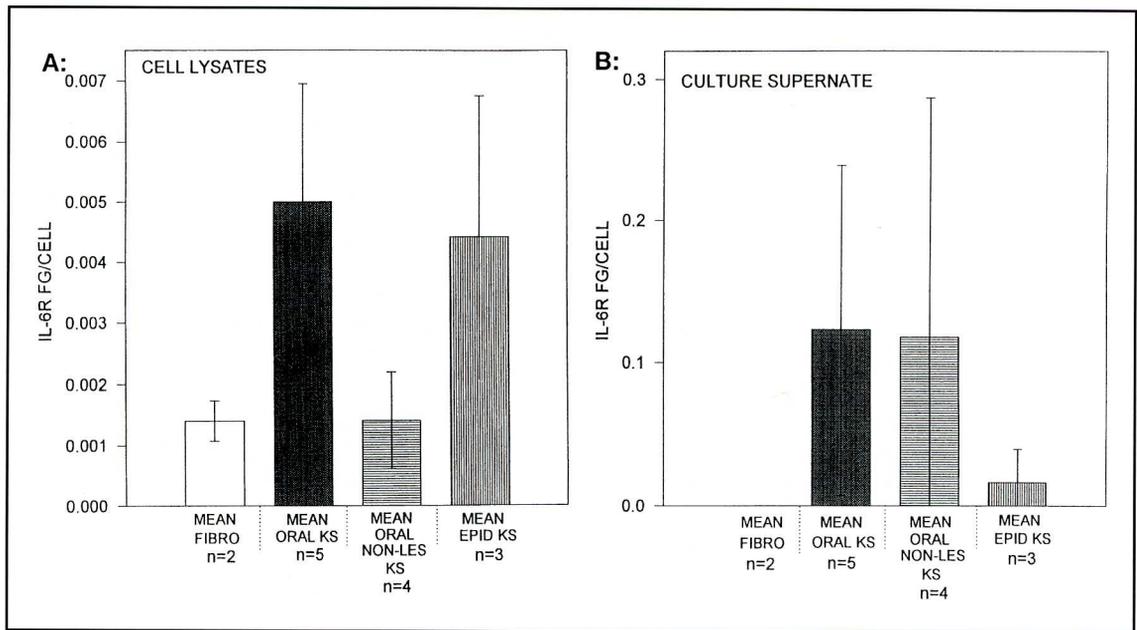
Fig. 2. ELISA quantitation of sIL6R concentrations: Cells and supernates were harvested at 80% confluence of culture at passage 5-7. Duplicate wells were averaged for each sample and mean values reported for 4 groups: Normal HIV-/KS- oral-derived fibroblasts (n=2)(FIBRO), Oral-derived AIDS-KS cell strains (n=5)(ORAL KS), Oral-derived non-lesional cell strains from HIV+/KS+ patients (n=4)(ORAL NON-LES KS), Epidermal derived AIDS-KS cell stains (n=3)(EPID KS). A: Cell lysates from harvested cells at a density of $1x10^8$ cell/ml. Statistical analysis (Kruskal-Wallis/MannWhitney) detected significant inter-group significant differences: Fibro vs Oral-derived KS p=0.01, Fibro vs Epidermal derived KS p=0.05, Oral- derived KS vs Non-lesional KS p=0.01, Epidermal derived KS vs Nonlesional KS p=0.05. B: Analysis of culture supernates. There were no statistically significant inter-group differences.

TTC CCA TGA G (51,55), NF-IL-6 GAC GTC ACA TTG CAC AAT CTT AAT AA (56) . Supershift analysis was carried out by modifying the EMSA technique by incubating the nuclear extract in separate reactions with 2 µl of one of the following rabbit polyclonal antibodies (Biomol, Plymouth Meeting, PA): 1. NF-κB p50, 2. NF-κB p65, 3. c-Rel, or 4. normal rabbit sera (The Binding Site, San Diego, CA). Incubation with sera/antisera was for 15 minutes at room temperature prior to addition of binding buffer and labeled oligonucleotide.

Statistical analysis

ELISA results were analyzed by grouping the cell strains into 4 categories: Normal fibroblasts (n=2), Oral derived-AIDS-KS cell strains (n=5), Non-lesional oral tissue from AIDS-KS patients (n=4), and Epidermal derived AIDS-KS cell strains (n=3). Statistical analysis consisted of the Kruskal-Wallis one way analysis of variance followed by Mann-Whitney nonparametric analysis of group mean values. Analysis with p < 0.05 were defined as statistically significant differences.

RESULTS

Cell Strain Expression of Gp-130 and IL-6R (α-Chain) Components of IL-6 Receptors

AIDS-KS and control cell strains in log phase growth were analyzed via ELISA for the presence of the gp-130 component of the IL-6 receptor (*Fig. 1*). Cell lysate analysis
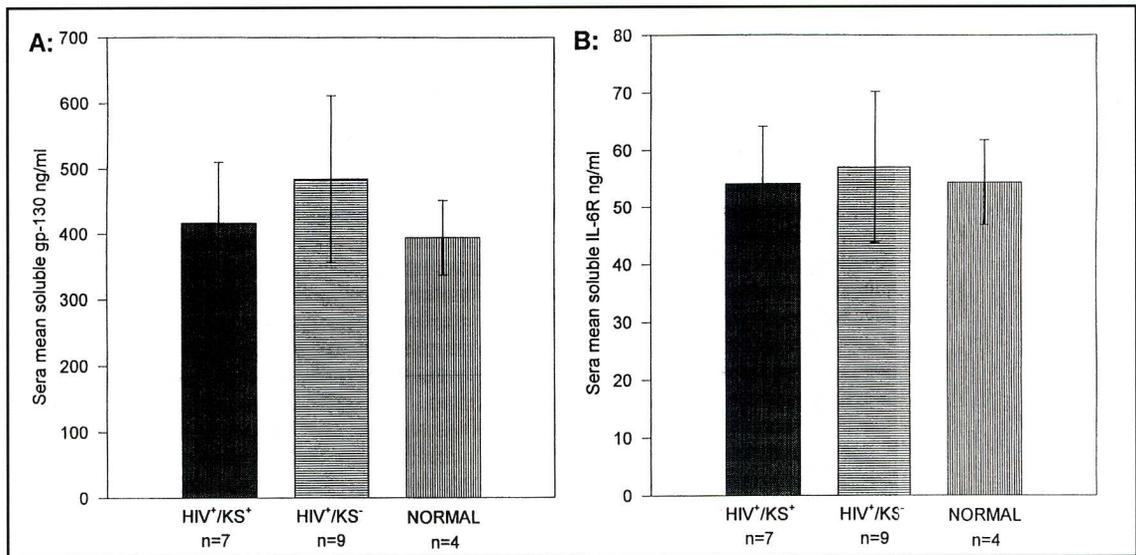
Fig. 3. ELISA quantitation of sera gp-130 and sIL6R concentrations: Sera samples were averaged from duplicate wells and means compared from three groups: HIV+/KS+ (n=7), HIV+/KS+ (n=9), Normal HIV-/KS- (n=4). A: Analysis for sera concentrations of gp-130. B: Analysis for sera concentrations of sIL6R (α chain). Statistical analysis (Kruskal-Wallis/Mann-Whitney) found no significant inter-group differences for either receptor component.

detected that AIDS-KS non-lesional cell strains contained significantly less gp-130 per cell than any of the other cells analyzed, inclusive of normal (HIV-) fibroblasts. While gp-130 was present in the supernates of all the cell strains analyzed, there were no other significant inter-group differences.

The alpha chain of the IL-6 receptor was found in cell lysates of all the cells analyzed (*Fig. 2*), with significantly higher concentrations/cell in all AIDS-KS strains compared to control strains of nonlesional cells from AIDS-KS patients and normal (HIV-) fibroblasts. There were no significant differences between IL-6R (α chain)/cell comparing normal fibroblast lysates with non-lesional strain lysate from AIDS-KS patients, or oral derived AIDS-KS stain lysates compared with epidermal derived AIDS-KS strain lysates. Soluble IL-6R (α chain) was not found in the supernates of normal fibroblasts. Analysis of culture supernates for soluble IL-6R (α chain) showed very high intra-group variability

resulting in large standard deviations of the mean concentrations/cell. As such, there were no significant intergroup differences when expressed as fg soluble-IL-6R/cell, and with only significant differences between normal fibroblasts and oral-derived AIDS-KS strains when quantitated as pg soluble-IL-6R/mg protein (p=0.044) (not shown).

Sera Analysis for Soluble Components of IL-6R

Sera from a population HIV+/KS+ (n=7), HIV+/KS- (n=9), and normal (HIV-) sera (n=4) were evaluated with ELISA for soluble gp-130 and IL-6R (α chain) (*Fig. 3*). Detectable concentrations of both components of the IL-6 receptor were found in all patients, with no significant inter-group differences.

Constitutive and Inducibility of Transcription Factors Ig-κb, IL-6-κB, NF-IL-6

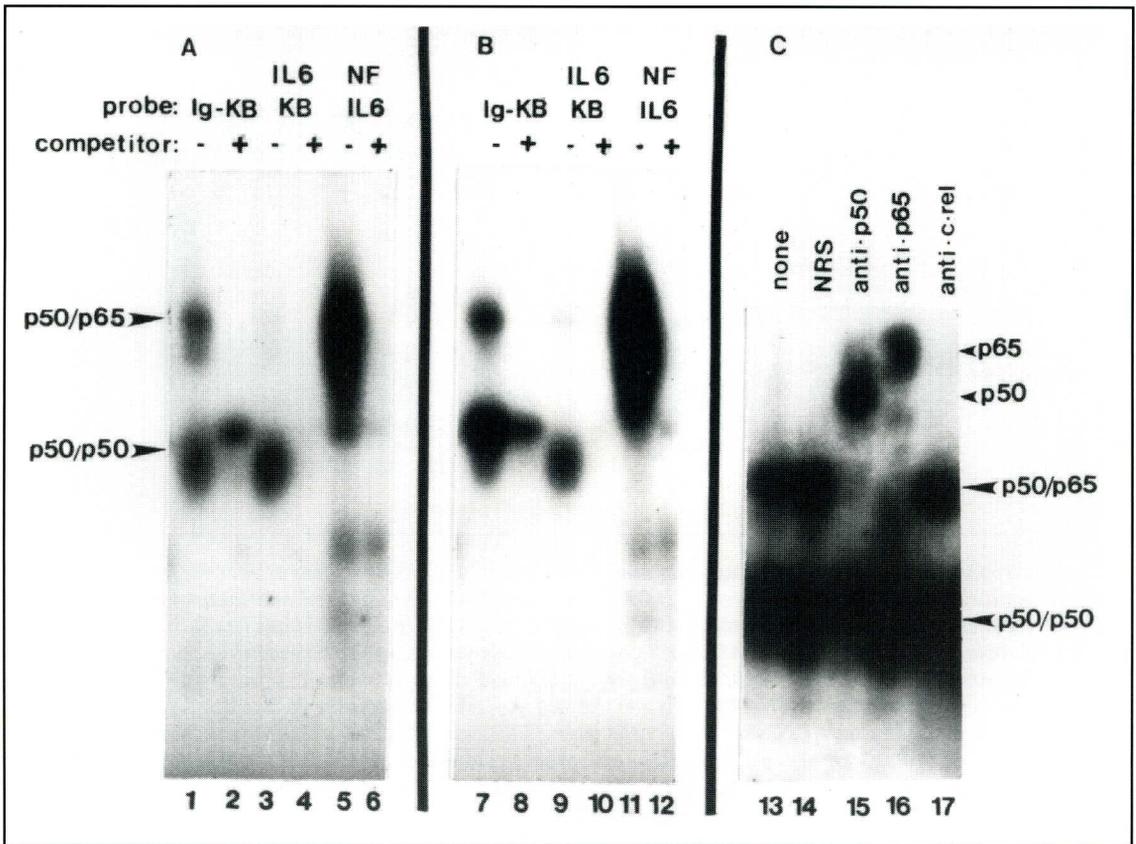Electrophoretic mobility shift assay was

Fig. 4. Electrophoretic mobility shift assay: log phase cultures: electrophoretic mobility shift assay (EMSA) was run as per (53) for: Oral-derived AIDS-KS stains (n=3), Epidermal-derived AIDS-KS cell stains (n=2), Oral-derived non-lesional cell strain from HIV+/KS+ patient (n= 1), and normal oral-derived fibroblasts from HIV-/KS-individuals (n=2). A: Representative Normal Fibroblasts, B: Representative AIDS-KS cell strain. Nuclear extracts from cells in log phase growth (15 μg) reacted with or without a 50x excess of competitive unlabeled oligonucleotides followed by reaction with P^{32} end-labeled oligonucleotide probes specific for Ig-κB, IL-6-KB, NF-IL-6. Samples were then separated in a 4 % non-denaturing polyacrylamide gel and exposed to X-ray film. There were no inter-group differences in the expression of high concentrations of all three transcription factors, with depletion of signals when competitors were added. Controls without nuclear extract run off the bottom of the gel and control with HeLa cell extract is the same as the shown cell strains. C: Super-shift EMSA to define the molecular species present in Ig-κB and IL-6-κB molecular complexes. EMSA was modified by reacting nuclear extracts with rabbit NF-κB component specific anti-sera before addition of labeled oligo. Lane 13: no sera, Lane 14: normal rabbit sera, Lane 15: anti-p50, Lane 16 anti-p65, Lane 17 anti c-rel. Nuclear extracts used are representative of all cell strains. Gel reveals that higher molecular weight band is p50/p65 complex, and lower molecular weight band is p50/p50 complex.

utilized to assess the constitutive expression of various transcription factors in oral (n=3) and epidermal (n=2) derived AIDS-KS cell strains and normal fibroblasts (n=2) and a cell strain derived from non-lesional oral tissue from an AIDS-KS patient (*Fig. 4*). Log phase cultures of all strains analyzed expressed detectable concentrations of NF-IL-6, Ig-κB, and IL-6-κB. Fifty fold excess of unlabeled target oligonucleotides were sufficient to inhibit binding to labeled target oligonucleotides while non-homologous (SP-1) competitors did not diminish specific reactivity with labeled target oligonucleotides (not shown). Supershift analysis confirmed the higher molecular weight species (slower
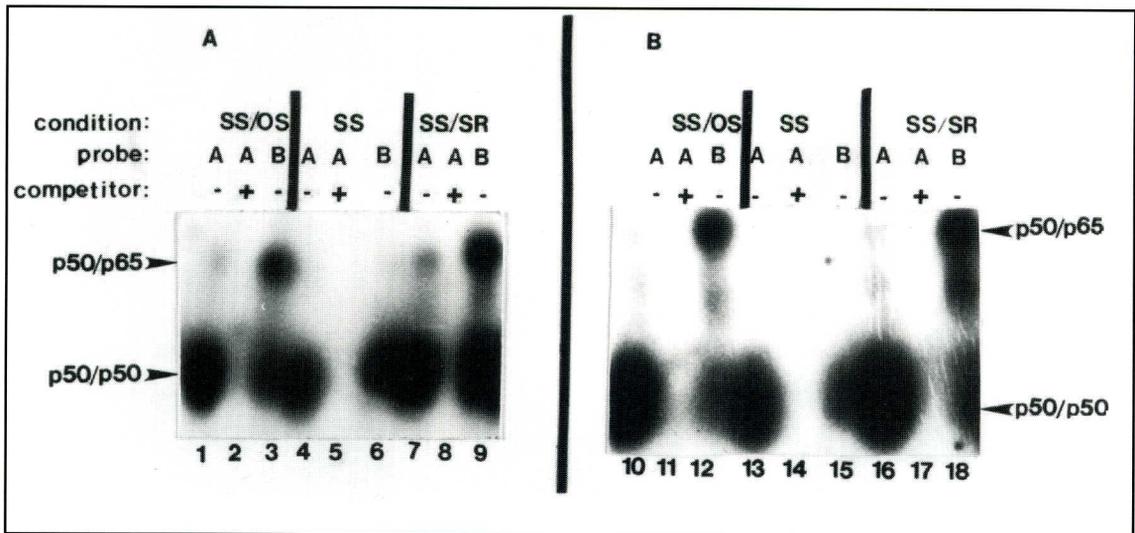
Fig. 5. Electrophoretic mobility shift assay: induction of components: EMSA was run as per (48) for: Oral- derived AIDS-KS stains (n=3), Epidermal-derived AIDS-KS cell stains (n=2) Oral-derived non-lesional cell strain from HIV+/KS+ patient (n=1), and normal oral-derived fibroblasts from HIV-/KS- individuals (n=2). A: Representative Normal Fibroblasts, B: Representative AIDS-KS cell strain. Cells were grown to 80% confluency, washed 3x with PBS and placed in M199 medium (without sera) for 5 additional days. At the end of the 5 day sera starvation, nuclear extracts were harvested (SS) or the cells were placed in medium containing 15 % FBS/ 5 % normal human sera for an additional 48 hours (SS/SR), or the cells were challenged with 200 ,μM H_2O_2 in M199 for one hour (SS/OS). Nuclear extracts were reacted with either of 2 probes/competitors: IL6-κB (A), or Ig-κB (B). Addition of 50-fold excess of unlabeled oligo (lanes 2,5,8,11,14,17) prevented the binding to the labeled oligo (lanes 1,4,7,10,13,16). Sera starvation (SS) caused depletion of Ig-κB and IL6-κB to negligible levels (lanes 4,6,13,15). Induction of sera-starved cultures with oxidative stress (SS/OS) or see-return (SS/SR) resulted in increased levels of Ig-κB (lanes 3,9,12,18) and IL6-κB (lanes 1,7,10,16) seen in log-phase cultures. There were no significant inter-group differences in the induction capacity of the cell strains.

migrating) as heterodimers of NF-κB components p50 and p65 (Rel-a), with the lower molecular weight species as p50 homodimers of NF-κB (Fig. 4c).

The capacity of Ig-κB and IL-6-κB to be induced in control fibroblasts and AIDS-KS derived strains was evaluated under conditions of 5-day sera starvation, 200 µM H_2O_2 challenge, and re-exposure to mitogens after sera starvation (sera-starve 5 day-sera return 48 hr) (Fig. 5). Preliminary studies showed that in order to detect a stimulation in Ig-κB and IL-6-κB expression, constitutive expression had to be minimized. We determined that a 5 day sera starvation of 80% confluent cultures was necessary to decrease constitutive expression but retain responsive cells. Significant inducibility

differences were not detected in oral or epidermally derived AIDS-KS strains, a non-lesional AIDS-KS strain, or normal fibroblasts. Upon sera starvation, all cell strains showed minimally detectable nuclear-translocation of IL-6-κB with significant increases after H_2O_2 treatment or return of mitogens. As was seen in log phase cultures (Fig. 4) and under induced conditions (Fig. 5) there were always stronger signals for Ig-κB than for IL-6-κB found in all cell stains.

DISCUSSION

These current investigations explored the hypothesis that our reported histological grade associated differences in the in vitro growth potential of AIDS-KS cell strains

(33,52) could be attributed to differential expression of the components of the IL-6 receptors. As mentioned, it has been disputed as to whether AIDS-KS cell strains contain IL-6 receptors (8,10,32,35). Based on this discrepancy, we speculated that our AIDS-KS strains derived from low histological grade lesions do not express functional IL-6 receptors, thereby explaining their inability to use autologously released IL-6. Furthermore, when cultured in an enriched medium, proliferation of cell strains derived from low grade AIDS-KS lesions would be sustained by the numerous growth factors present in the medium. However, when in medium only supplemented with low sera concentrations, proliferation would cease due to their inability to utilize autologously produced IL-6 and absence of other proliferation supporting growth factors. Our results, however, failed to support this hypothesis, for we found no significant histological grade associated differences in the expression of either the α or ß chains of the IL-6 receptor in AIDS-KS derived cell strain lysates. The biological consequences of the observed increased expression of IL-6 receptors in AIDS-KS lesional derived cell strains relative to strains from non-lesional oral tissue of AIDS-KS patients or normal oral-derived fibroblasts imply a growth advantage to the lesional AIDS-KS cells. It has been clearly established that AIDS-KS cell strains release more IL-6 than control non-lesional cells (7,10,11, 21,30,33). As such, this implies the potential for autologous stimulation of AIDS-KS strains via IL-6. Such investigations are complicated by the question as to whether quantitative differences in receptors have a biological significance if all cells express the receptor. To address this issue further investigations would be required to assess the degree of receptor saturation in cell populations. In addition, future investigations need to explore whether there are similar quantitative differences in the expression of IL-6 receptors in lesional vs nonlesional AIDS-KS tissue *in vivo*.

Quantitative comparisons of soluble components of the IL-6 receptor in the culture supernates indicated only significant differences in concentrations of the α chain between normal fibroblasts (which do not release detectable concentrations) and AIDS-KS cell strains derived form high grade lesions when expressed in pg/mg total protein in the supernate. The biological functions of soluble IL-6 receptor components include: 1. sIL-6R (α chain) binding IL-6 and complexing with cell-bound gp-130 (58-61,63,64), 2. soluble gp-130 binding soluble IL-6R (α chain):IL-6, subsequently binding to gpl30 positive cells (63), 3. either sIL-6R(α chain) or gpl30 can bind soluble IL-6 or sIL6R:IL6 complexes (respectively) and reduce the binding sites for interaction with cell-bound receptors (58-61,63,64). In our *in vitro* model where cells express both receptor components cell bound, it is very difficult to predict what, if any, effect soluble receptor components have. To assess this definitively, flow cytometry analysis would be necessary to determine if there are sub-populations of gp-130+/α chain- cells where soluble IL-6R (α chain) could potentiate positive signaling to these otherwise IL-6 non-responsive cells.

These investigations utilized ELISA analysis to quantitate receptor concentrations in whole cell lysates. Perhaps use of ELISA designed for the soluble receptor components is not appropriate to quantitate intracellular/ membrane bound receptors. Whereas there are techniques more appropriate for quanti-tating membrane bound receptors (receptor labeling), ELISA analysis provides a straightforward method to quantitate both cell associated and released receptor concentrations. Because the ELISA is based on detection of soluble IL-6R (α-chain) and soluble gp-130 (ß-chain), the capture antibodies are based on extracellular epitopes of the proteins. As such, the same antibodies would still function to recognize the membrane bound forms of the receptors found in the cell lysates. Furthermore, the fact that measurement of receptor

components in whole cell lysates is complicated by the presence of cytokine:cytokine receptor complexes applies equally to the measurement of soluble cytokine receptors in culture supernates. Thus, despite possible complications with the use of ELISA to analyze cell associated receptor concentrations, the use of this technique is, nonetheless, valid for direct comparisons of both intercellular and soluble receptor components.

Soluble IL-6R (α-chain) and gp-130 have both been detected in sera (60,63,65) and/or urine (α chain only) (62) of normal individuals. It is known that soluble forms of both components are lacking the intracellular and cytoplasmic domains of the receptors, but it is not completely known whether these components are from: 1. unique RNA transcripts, 2. proteolytic release of membrane bound receptors, or 3. whether they are prematurely terminated transcripts of the receptors, resulting in truncated proteins (64,66). The biological consequences of sera soluble IL-6 receptor components are the same as previously mentioned for culture medium soluble IL-6 receptors. Elevated sera concentrations of soluble IL-6R (α chain) have been reported in HIV seropositive (65) and monoclonal gammopathy patients (60). We speculated as to whether there were quantitative sera concentration differences in AIDS patients with and without KS for the soluble forms of IL-6 receptors. It has been suggested that both OSM and IL-6 are important *in vivo* growth factors of KS cells (10,13,30,67). We have previously reported that there were no significant differences in sera cytokine concentrations between AIDS patients with and without KS (33). However, it is possible that sera analysis for OSM and IL-6 may have been complicated by high concentrations of sera soluble cytokine receptors. It is possible that if OSM is an essential growth factor for KS, then increased concentrations of sera soluble gp-130 would function to bind OSM to help potentiate cell stimulation of gp-130+ KS cells. As such, one might then expect elevated concentrations of

gp-130 in the sera of HIV+/KS+ patients. Our analysis of a population of normal and AIDS patients both with and without KS did not indicate any significant differences in sera concentrations of IL-6 receptors. These results contradict the previous report of elevated soluble IL-6R (α chain) in AIDS patients (65) likely due to the therapy our patients were receiving and/or patient variability. Overall, these results are in line with previous findings that any role cytokines play *in vivo* is likely a local, rather than systemic, phenomenon (33,67,68).

Our interest in the expression of NF-κB in AIDS-KS derived cell strains was based on several factors: 1. The HIV tat gene codes for a protein (Tat) which interacts with TAR (Tat responsive elements) RNA sequences in the HIV *LTR* region to liberate NF-κB mediated HIV transcription (38,39). Since the Tat protein has been shown to be mitogenic for AIDS-KS cell strains (22,28,40), one might speculate that AIDS-KS cell strains have a similar Tat mediated stimulation of NF-κB resulting in sustained upregulation of NF-κB responsive genes. 2. Altered capacity to regulate NF-κB gene transactivation has been associated with diseases such as ataxia telangiectasia where there is altered capacity of IκB components to form the cytoplasmic NFκB: IκB complexes (41). In addition, it has been demonstrated that alteration of regulation of NF-κB/IκB can potentiate a malignant phenotype both *in vitro* (42-46) and *in vivo* (42,43,45). The potentiation of the malignant phenotype is attributed to the presence of NF-κB responsive sequences in genes for proteins associated with malignant phenotypes such as extracellular proteases, angiogenic factors, and adhesion molecules (42-46). Based on the association of altered NF-κB expression with other diseases and malignancies, it is a reasonable hypothesis that similar defects may occur in AIDS-KS. 3. Oxidative stress (via H_2O_2 treatment) has been associated with upregulation of NF-κB gene transactivation (47-50). Thus, in T-lymphocytes a 30% decrease in glutathione

concentrations results in: decreased Ca^{+2} mobilization, increased tyrosine phosphorylation, and induction of AP-1 and NF-κB (49,50). We have reported that AIDS-KS cells have reduced capacity to respond to oxidative stress as indicated by altered nucleotide pools, decreased glutathione, and inability to upregulate catalase (15,16). 4.) NF-κB is a transcription factor for numerous cytokines inclusive of IL-6 (36,37,51). AIDS-KS strains are known to have unregulated expression of IL-6 (7,10,11,21,30,33), and it is possible that AIDS-KS cells have altered capacity to regulate NF-κB gene transactivation.

We found that in comparison to normal oral derived fibroblasts there was no difference in the constitutive, oxidative stress, or mitogen stimulated expression of NF-κB in AIDS-KS derived cell strains. Also, the histological grade associated differences in the growth potential of AIDS-KS strains cannot be explained by differences in expression of NF-κB. In addition to analyzing the expression of Ig-κB, we analyzed the expression of IL-6-κB. Our results indicate detection of both species of NF-κB in control and AIDS-KS cell strains. The differences in the signal strength of IL-6-κB, and Ig-κB in the EMSA are likely attributable to the assay being optimized for Ig-κB in terms of oligo and buffer conditions. This relative difference in signal strength should not be interpreted as a concentration difference in species of NF-κB. To date there have been no reports of species of NF-κB that will only bind to specific promoter NF-κB sequences (37,51). Thus, the same NF-κB protein will hybridize equally to promoter regions for Ig as IL-6.

NF-IL-6 is another transcription factor for IL-6, and like NF-κB, has the potential to transactivate various cytokine genes either independently or in association with NF-κB (69). Regardless of the growth conditions in these current investigations (including oxidative/environmental stress), there were always high levels of NF-IL-6, with no difference in the constitutive or inducible expression of NF-IL-6 in AIDS-KS cell strains or normal oral derived fibroblasts. Because NF-IL-6 is not oxidative/environmental stress responsive, as is NF-κB (47,48), stress induced increased levels of NF-IL-6 are not expected. These investigations comparing transcription factor expression (Ig-κB, IL-6-κB, and NF-IL-6) in AIDS-KS and control strains are based solely on the nuclear presence of the transcription factor and its capacity to bind to the target oligonucleotides. Future studies need to address whether there are histological grade associated differences in AIDS-KS cell strains' capacity to transactivate transfected transcription factor responsive-reporter gene constructs as compared with normal non-lesional cells. Such investigations are imperative to clarify if the transcription factors present in the nuclear fraction are biologically active.

Whereas these investigations have not conclusively defined the reason for histological grade associated differences in the growth capacity of AIDS-KS cell strains, they have eliminated two potential mechanisms for this phenomenon. Thus, there are no grade associated differences in the expression of the components of the IL-6 receptor or in the constitutive or inducibility of NF-κB transcription factors. Moreover, these investigations failed to find significant differences in the sera levels of soluble IL-6 receptor component concentrations in HIV+/KS+ and HIV+/KS- populations, further supporting the fact that cytokine contribution to stimulate AIDS-KS cell proliferation is a local rather than systemic effect (33,67,68).

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