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AIDS-associated Kaposi's sarcoma (KS) cells express oncostatin M (OM)-specific receptor but not leukemia inhibitory factor/OM receptor or interleukin-6 receptor. Complete block of OM-induced KS cell growth and OM binding by anti-gp130 antibodies.

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Research Article

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AIDS-associated Kaposi's Sarcoma (KS) Cells Express Oncostatin M (OM)-specific Receptor but Not Leukemia Inhibitory Factor/OM Receptor or Interleukin-6 Receptor

Complete Block of OM-induced KS Cell Growth and OM Binding by Anti-gp130 Antibodies

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Abstract

Oncostatin M (OM), which shares functional similarity and structural homology to leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), functions as a potent growth factor for AIDS-associated Kaposi's sarcoma-derived cells (AIDS-KS cells). OM was also suggested to bind to the LIF receptor (LIF/OM receptor), which consists of a signal transducing subunit for LIF and IL-6 (gp130) and a LIF receptor α-subunit. Recent studies indicate that IL-6 has growth-stimulating activity for AIDS-KS cells. However, we find that AIDS-KS cell growth is exclusively induced by OM and not by LIF or IL-6. We also observed the lack of binding properties of AIDS-KS cells for LIF and IL-6. Scatchard plots revealed the existence of two affinity classes of OM receptor sites on AIDS-KS cells, with K_d values of 6–12 pM (high affinity) and 521–815 pM (low affinity). In competition binding studies, we find that the OM-specific receptor, but not the LIF/OM receptor, contributes to the OM-specific growth stimulation of AIDS-KS cells. We also noted that anti-gp130 antibodies can completely abolish OM-induced growth stimulation of AIDS-KS cells as well as OM binding to AIDS-KS cells. PCR amplification clearly revealed high levels of gp130 expression in AIDS-KS cells, while the transcript of LIF receptor α-subunit or IL-6 receptor α-subunit was not observed. Therefore, we conclude that *(a)* AIDS-KS cells express the OM-specific receptor with high and low affinity, but not the LIF/OM receptor; *(b)* gp130 on AIDS-KS cells plays a key role in OM binding and signaling on the OM-specific receptor; and *(c)* the lack of biological response of AIDS-KS cells to IL-6 and LIF can be explained by the absence of the IL-6 and LIF/OM receptors. All this evidence shows the correlation of OM-specific biological activity with expression of the OM-specific receptor and the involvement of gp130 on this receptor, as based on findings in in vitro growth assays and binding experiments for AIDS-KS cells. (*J. Clin. Invest.* 1995. 96:1319–1327.) **Key words: cytokine receptor • growth factor • biological signaling • binding sites • growth inhibition**

Introduction

Kaposi's sarcoma (KS)[1] is a multicentral proliferative lesion of unknown etiology and unclear pathogenesis (1). The most common form of KS is frequently associated with HIV infection (AIDS-KS) (2) and with immune suppression due to other causes (3). KS is an unusual neoplasm with unique features: a high incidence of spontaneous remission, rapid occurrence of multifocal lesions, and lack of metastasis (4). KS lesions also have a complex histology characterized by a high degree of neovascularization, edema, and proliferation of spindle-shaped cells (KS cells) (5). Development of AIDS-KS lesions has been reported to consist of multiple steps and interactions of several diverse cytokines (6–9), suggesting that KS is not a real tumor but rather a cytokine-mediated proliferative disease. Recently, our group and others reported that a growth regulatory protein, oncostatin M (OM), is a potent mitogen for AIDS-KS cells in culture. Hence, OM may play an important role in the initiation and/or progression of AIDS-KS lesions (10–12). However, little is known of OM receptor sites on AIDS-KS cells, through which OM exerts its growth-stimulating activity. Characterization of OM receptor sites on AIDS-KS cells will provide a better understanding of possible roles of OM in growth regulation of these cells and pave the way toward development of new drugs.

OM, a 30-kD glycoprotein, was originally identified from activated human T cells and a differentiated histiocytic leukemia cell line, based on the potential to inhibit the growth of melanoma cells (13–15). Subsequently, OM was characterized as a growth regulator for certain tumor- and non-tumor-derived cell lines. Its pleiotropic biological activities encompass stimulation or suppression of pathways leading to differentiation or proliferation, and induction of other cellular target genes (16–19). Several groups reported that OM is a member of a cytokine family that includes leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), because OM shares structural homology and functional similarity to LIF and to IL-6 (20–23). Recently, it

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1. *Abbreviations used in this paper:* AIDS-KS, AIDS-associated Kaposi's sarcoma; BES, 2-[bis(2-hydroxyethyl)amino] ethanesulfonic acid; CHO, Chinese hamster ovary; CM, conditioned medium; ds, double strand; DSS, disuccinimidyl suberate; KS, Kaposi's sarcoma; LIF, leukemia inhibitory factor; OM, oncostatin M; rhIL-6, recombinant human interleukin-6; rhLIF, recombinant human leukemia inhibitory factor; rhOM, recombinant human oncostatin M.

was proposed that LIF and OM bind with high affinity to the same receptor (LIF/OM receptor), which consists of a signal transducing subunit for LIF and IL-6 (gp130) and a LIF binding subunit (LIF receptor α-subunit) (24). The roles of these subunits on the LIF/OM receptor in OM binding and its signal transduction have yet to be determined, but the overlapping biological activities of these cytokines may be explained by their sharing of the LIF/OM receptor. In contrast, several other biological responses have been reported to be exclusively induced by OM (16, 17), a finding that suggests existence of an OM-specific receptor and its importance in OM-specific signaling. We asked whether AIDS-KS cells carry a LIF/OM receptor or an OM-specific receptor. gp130 was reported to be capable of binding OM with low affinity (24–26). If OM-specific biological responses correlate with expression of the OM-specific receptor, it needs to be determined if the OM-specific receptor includes gp130 as a binding subunit and/or a signal transducting subunit.

Miles et al. reported that IL-6 is also a growth factor for AIDS-KS cells, determined by using the antisense oligonucleotide for the coding region of IL-6 (27). However, we obtained evidence that the growth-stimulating response of AIDS-KS cells is not induced by either LIF or IL-6. The fundamental question is whether the difference in biological response among OM, IL-6, and LIF arises from the specificity in receptor expression or relates to differences in postreceptor pathways on AIDS-KS cells. We report here that AIDS-KS cells express the OM-specific receptor but not the LIF/OM receptor or the IL-6 receptor. The absence of the latter two receptors on AIDS-KS cells results in a lack of biological effects of IL-6 and LIF on KS cells. Furthermore, we show that the OM-specific receptor on AIDS-KS cells includes gp130 as an OM binding subunit and that gp130 is an essential component for the signaling of OM-induced growth stimulation of AIDS-KS cells.

Methods

Cells and reagents. AIDS-KS cells were developed from the pleural effusion of lung KS (AIDS-KS–USC22, -KS1, -KS2 [or N521J], -KS4, -KS5, -KS6, -KS7, -KS9, -KS11, and -KS12), lung KS (AIDS-KS3, -KS8, and -KS13), oral mucosa KS (AIDS-KS10B), and skin KS (AIDS-KS14) of HIV-1 infected patients. AIDS-KS–USC22 cells were established in our laboratory, using previously mentioned methods (6, 10), and the other 14 isolates were developed in the Laboratory of Tumor Cell Biology (National Cancer Institute, National Institutes of Health, Bethesda, MD). AIDS-KS cells were maintained in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD), supplemented with 15% FBS (Sigma Chemical Co., St. Louis, MO) and conditioned medium (CM) from human OM-expressing Chinese hamster ovary cells (CHO; CCL61; American Type Culture Collection, Rockville, MD) to a final OM concentration of 15 ng/ml. CHO cells were cultured in Ham's F12 medium supplemented with 10% FBS. Recombinant human OM, LIF, and IL-6 (rhOM, rhLIF, and rhIL-6) were purchased from R & D Systems (Minneapolis, MN). Mouse anti-human gp130 monoclonal antibodies (GPX7, GPX22, and GPZ35) were prepared as described previously (26).

AIDS-KS cell growth assay. AIDS-KS cells were incubated in triplicate in gelatinized 24-well plates (0.5 ml/well) in medium (RPMI 1640 supplemented with 15% FBS), with or without various test factors, at an initial cell density of 3×10^3 cells/well. Culture medium was changed every 3 d, and cells were counted on the day 6 of culture using a particle counter (Coulter Electronics Inc., Hialeah, FL).

Isolation of human OM cDNA. Double strand (ds) cDNA was synthesized from poly $(A)^+$ RNA obtained from human CD_4^+ T cells (38-10 cells) reported to express OM highly and constitutively (10, 11). To isolate cDNA containing the entire coding region of OM, PCR amplification was performed using the ds cDNA of 38-10 cells as a template and a single pair of the following oligonucleotides as a set of primers. The forward primer contains the nucleotide sequence 5′-GCCGGAGCACGGGCACCCAGCATGGGGGTACT-3′, corresponding to amino acids 1–4 at the NH_2 terminus of OM and 21 nucleotides of the 5′ untranslated region. The reverse primer contains 5′-CTCGAG-GCTACCGGGGCAGCTGTCCCCTGGT-3′, which is the reverse complement of the nucleotide sequence encoding amino acids 246–252, followed by a stop codon at the COOH terminus, and 7 nucleotides of the 3′ untranslated region. The resulting PCR product was inserted into the pCRII vector (In Vitrogen, San Diego, CA) at the EcoRV site at which 3′ terminal thymidine overhangs are added to both ends. The resulting plasmid was designated pCRII-OM.

Establishment of human OM-expressing CHO cells. To construct a human OM-expression vector in mammalian cells, the human OM cDNA fragment containing the entire coding region was excised from pCRII-OM and inserted into the mammalian expression vector pRc/CMV (In Vitrogen) between HindIII and XbaI sites. The resulting OM-expression vector, pRc/CMV-OM, was transfected into CHO cells using Lipofectin reagent (GIBCO BRL), according to the manufacturer's instructions. OM-expressing clones were selected with Geneticin (GIBCO BRL). Most of the selected clones produced OM in medium at a range of 0.5–1.5 μg/ml, as confirmed biologically and immunologically by AIDS-KS cell growth assay, ELISA, and Western blot analysis (data not shown).

RNA preparation and poly $(A)^+$ selection. Total RNA was prepared from 38–10 cells and AIDS-KS cells by guanidine isothiocyanate disruption of cells and by centrifugation through 5.7 M cesium chloride. Poly $(A)^+$ RNA was selected from total RNA fraction by passing through an oligo (dT) cellulose spum column purchased from Pharmacia LKB Biotechnology (Piscataway, NJ).

PCR amplifications for human gp130, LIF receptor α-subunit, and IL-6 receptor α-subunit. To prepare templates for positive controls, ds cDNA fragments for human gp130, LIF receptor α-subunit, and IL-6 receptor α-subunit were generated by PCR amplifications using cDNA derived from human liver (for gp130 and LIF receptor α-subunit) and from U937 (for IL-6 receptor α-subunit), respectively. The primers used were the following: 5′-AAGGATGGTCCAGAATTCAC-3′ and 5′-CCTTCACTGAGGCATGTCGC-3′ (for gp130); 5′-ATGTAT-GTGGTGACAAAGGA-3′ and 5′-GTAAAGGACCACCCTCCTCC-3′ (for LIF receptor α-subunit); and 5′-ATGTGCGTCGCCAGT-AGTGT-3′ and 5′-GGGAAGAAGTAGTCTG TATT-3′ (for IL-6 receptor α-subunit). The respective PCR products were inserted into the pCRII vector. The resulting vectors were referred to as pCRII-gp130, pCRII-LIFRec and pCRII-IL6Rec, which carry 960-bp, 791-bp, and 826-bp cDNA fragments, respectively. The ds cDNA fragments were excised from the respective vectors and then included as templates for positive controls in PCR amplifications using the corresponding primer sets.

^{35}S-labeling of recombinant OM, LIF, and IL-6. ^{35}S-labeling of purified recombinant OM, LIF, and IL-6 was performed using the [^{35}S]-sulfur-labeling reagent, [^{35}S]LR (Amersham Corp., Arlington Heights, IL), according to the manufacturer's instructions. Each protein (2.5 μg) in 10 μl of 0.1 M borate buffer (pH 8.5) was added to the reaction vessel containing [^{35}S]LR (>800 Ci/mmol) and then incubated for 30 min on ice. The reaction was terminated with 100 μl of 0.2 M glycine in 0.1 M borate buffer (pH 8.5). ^{35}S-labeled proteins were purified by passage through a Sephadex G-25 column (Pharmacia) for separation from free [^{35}S]. Purity and molecular weight of ^{35}S-labeled cytokines were examined by SDS-PAGE and fluorography. ^{35}S-labeled OM also possessed full growth-stimulating activity on KS cells. Preparation of ^{35}S-labeled OM, LIF, and IL-6 used for our experiments had specific activities of 3.6×10^{14}, 3.0×10^{14}, and 3.3×10^{14} cpm/mmol, respectively.

In vitro transcription and translation. To generate a plasmid that can be used for the synthesis of a ^{35}S-labeled mature form of human

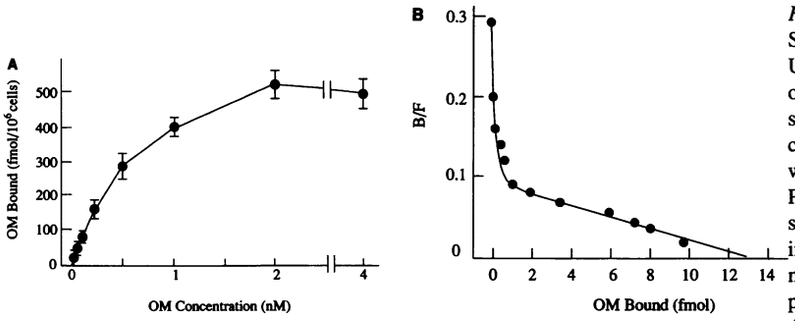

Figure 1. Binding of ^{35}S-labeled OM to AIDS-KS cells and Scatchard plot analysis. (*A*) Saturation curve: AIDS-KS–USC22 cells (2.5×10^4 cells) were examined for binding of increasing concentrations of ^{35}S-labeled OM, as described in Methods. Total binding was determined in duplicate wells. Nonspecific binding was determined in duplicate wells in the presence of 100-fold excess of unlabeled OM. Points represent specific binding, which was determined by subtraction of nonspecific from total binding. Specific binding accounted for 75–95% of total binding. Data represent mean from duplicate measurements in five independent experiments ($n = 10$). (*B*) Computer-fit Scatchard plot: the data from equilibrium binding experiments of ^{35}S-labeled OM on AIDS-KS–USC22 cells from five independent experiments were analyzed using the LIGAND computer program (29) and re-expressed as a Scatchard plot. Values are means±SD from duplicate determinations in five separate experiments ($n = 10$). Equilibrium binding experiments and Scatchard analysis were also performed with AIDS-KS3 and -KS10B cells in duplicate in two independent experiments for each ($n = 4$); essentially similar results were obtained. Binding parameters (±SD) of two hypothetical sites are given for each of three AIDS-KS cell isolates in the text.

OM in an in vitro transcription/translation system, PCR amplification was performed using two specially designed oligonucleotides as a set of primers and ds cDNA derived from OM–expressing 38–10 cells as a template. The forward primer contained the nucleotide sequence 5′-CATATGGCGGCTATAGGCAGCTGCTCGAAAGA-3′ corresponding to the sequence of amino acids 26–34 and the preceding ATG codon, which introduces an artificial ATG initiation codon upstream of the NH_2 terminus of the mature OM (mOM). The reverse primer contained 5′-GGATCCCTCGAGGCTACCGGGGCAGCTGTCC, which is the reverse complement of the nucleotide sequence encoding amino acids 248–252 followed by a stop codon at the COOH terminus, and 7 nucleotides of the 3′-untranslated region. The resulting PCR fragment, which carries the nucleotide sequence encoding the mOM, was inserted into the pCRII vector at EcoRV. The resulting vector, pCRII-mOM, was used for in vitro transcription and translation of the mOM.

mOM-specific mRNA was synthesized from linearized pCRII-mOM by an in vitro transcription system (Ambion, Austin, TX) with SP6 RNA polymerase, according to the manufacturer's instructions. Then the mOM mRNA was translated in a nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of [^{35}S]methionine (> 800 Ci/mmol; Amersham), according to Promega's instructions. To remove free [^{35}S]methionine, the products were passed through a Sephadex G-50 column. The specific activity of ^{35}S-labeled OM was estimated to be 2×10^{15} cpm/mmol. Molecular weight and purity of ^{35}S-labeled OM were confirmed by SDS-PAGE and fluorography. ^{35}S-labeled OM possessed full growth-stimulating activity on AIDS-KS cells.

Binding assay. AIDS-KS cells were grown to confluence (2.5×10^4 cells/well) in duplicate (0.5 ml) in 24-well plates. The plates were washed three times with binding buffer (DME containing 50 mM BES, pH 6.8, and 0.1% BSA) and incubated at 37°C for 4 h in binding buffer to allow for endocytosis of cellular receptors that bound OM from the culture medium. After three additional washes, the cells were incubated with various titrated concentrations of ^{35}S-labeled OM starting at 4 nM in a volume of 0.2 ml of the same buffer. Binding reactions were carried out for 3 h at room temperature. Nonspecific binding was measured in the presence of 100-fold excess of unlabeled OM. The specific binding was calculated by subtracting nonspecific binding from total binding (duplicate determinations). At the end of the incubation period, the cells were washed four times with binding buffer to remove unbound ^{35}S-labeled OM and then trypsinized. Cell-bound radioactivities were measured in Aquasol (New England Nuclear, Boston, MA) using a scintillation counter. Variation between duplicate determinations was generally less than 10%. The dissociation constant and an average number of binding sites per cell were calculated by Scatchard plot analysis using saturation binding data (28). Data from binding experiments with AIDS-KS cells were also analyzed using the LIGAND computer program (29). For competition binding studies, the unlabeled cytokines were serially titrated, starting at a concentration of 0.5 nM. ^{35}S-labeled OM was added at a constant concentration of 0.5 nM. ^{35}S-labeled and unlabeled cytokines were mixed before adding to AIDS-KS cells. Binding reactions were incubated for 4 h at room temperature in a volume of 0.2 ml of binding buffer. Binding assays for LIF and IL-6 on KS cells followed the same protocol as the OM-binding assay.

Cross-linking experiment. AIDS-KS cells were grown to confluence in 24-well plates (2.5×10^4 cells/well) and washed three times with the same buffer. After incubation at 37°C for 4 h in binding buffer, the cells were washed three times with binding buffer and incubated with ^{35}S-labeled OM at a concentration of 1 nM for 3 h at room temperature. To demonstrate specific competition, 100 nM of unlabeled OM was added to ^{35}S-labeled OM. Following the binding reaction, the cells were washed four times with binding buffer, once with PBS, and incubated in PBS at room temperature (0.2 ml/well). Disuccinimidyl suberate (DSS; Pierce Chemical Co., Rockford, IL) was added from freshly prepared concentrated stock solution in dimethyl sulfoxide to give a final concentration of 2 mM. The cells were incubated on ice for 15 min, washed once with binding buffer, and solubilized with 0.1 ml of SDS–sample buffer. The samples were boiled for 5 min and electrophoresed in 10% SDS-PAGE followed by fluorography.

Results

Binding experiments of ^{35}S-labeled OM to AIDS-KS cells: number of receptor sites and affinity. To identify the OM receptor on AIDS-KS cells, binding experiments of ^{35}S-labeled OM to AIDS-KS cells were performed (Fig. 1). Radiolabeling of OM with [^{35}S] was carried out using an in vitro transcription/translation system and an external labeling system. Both preparations had a similar level of binding and growth-stimulating activities for AIDS-KS cells (data not shown). We usually used in vitro–translated ^{35}S-labeled OM, especially to confirm the existence of a high affinity OM receptor. Since AIDS-KS cells were maintained in CM from OM-expressing CHO cells, the OM receptor may be occupied by OM from CM. To allow for endocytosis of bound OM and the occupied OM receptor, the cells were incubated for 4 h at 37°C prior to the binding experiments. As shown in Fig. 1*A*, AIDS-KS–USC22 cells specifically bound ^{35}S-labeled OM, in a dose-dependent fashion, and reached a plateau at 2 nM of ^{35}S-labeled OM, when the cells were incubated with increasing concentrations of ^{35}S-labeled OM. The extent of specific binding generally constituted 75–95% of the total binding in the absence of unlabeled OM. To determine the dissociation constant for ^{35}S-labeled OM binding and an average

number of receptor sites per cell, a Scatchard plot (28) was constructed from saturation binding data obtained in Fig. 1 *A* (Fig. 1 *B*). The plot was a curvilinear curve and best fit by a curve generated for a two-binding-site model, which revealed two affinity classes of OM receptors on AIDS-KS–USC22 cells. ^{35}S-labeled OM bound to KS cells with a higher affinity at lower ^{35}S-labeled OM concentrations. Using a two-receptor model, dissociation constants (K_d values) of the two hypothetical sites of 7±1 pM (high affinity) and 720±60 pM (low affinity) were obtained for AIDS-KS–USC22 cells. These estimates of affinity on AIDS-KS cells were in the range of estimates for other OM responsive cells (24,30). Average numbers of high and low affinity receptors of OM were 8,700±300 and 340,000±100,000 per cell, respectively; the values are high when compared with receptor levels on other OM-responsive cells (24,30). Essentially similar data were obtained in experiments done with AIDS-KS10B and -KS3 cells. The calculated K_d values of high and low affinity were 12±2 pM and 815±5 pM for KS10B cells and 6±1 pM and 521±90 pM for KS3 cells. The average numbers of high and low affinity sites per cell were 9,100±400 and 410,000±70,000 for KS3 cells and 12,300±400 and 315,000±30,000 for KS10B.

Correlation of OM-induced biological activity with OM binding. To evaluate the biological relevance of ^{35}S-labeled OM binding on AIDS-KS cells, the concentration of ^{35}S-labeled OM that results in significant binding was compared with that responsible for AIDS-KS growth-promoting activity (Fig. 2). Serial twofold dilutions of ^{35}S-labeled OM were incubated with AIDS-KS cells (KS-USC22, KS10B, and KS3) in the growth assay to measure the response to ^{35}S-labeled OM. The specific biological activity of ^{35}S-labeled OM was similar to that of recombinant OM prior to radiolabeling (data not shown). Data from these experiments were compared with the binding curve of ^{35}S-labeled OM on AIDS-KS cells. The concentration of ^{35}S-labeled OM that can promote half-maximal AIDS-KS growth response was 0.01–0.02 nM. In contrast, half-maximal saturation of ^{35}S-labeled OM receptor required a concentration of 0.45–0.65 nM. These data show that biological effects of OM on AIDS-KS cells are achieved at partial receptor occupancy, which means that KS cells have a high proportion of spare receptors for OM. For many cytokines, this difference was noted between the concentration required to achieve maximal biological response and that required for maximal binding (31). The concentration of ^{35}S-labeled OM giving a half-maximal biological response corresponded to 55–60% saturation of the high affinity receptor and 1–4% of low affinity receptor. Our findings indicate that occupancy of a high affinity receptor more than a low affinity receptor is important for growth-stimulating activity of OM. There were no significant differences among data obtained from experiments done with three different isolates of AIDS-KS cells. Therefore, it seems likely that OM-induced stimulation of AIDS-KS cell growth correlates with expression of the high affinity receptor for OM.

Specificity of biological activity and binding of OM to AIDS-KS cells: lack of growth stimulating effects and binding properties of IL-6 and LIF on AIDS-KS cells. OM is structurally and functionally related to LIF and IL-6 (20–23). Previous work revealed that IL-6 as well as OM have growth stimulating effects for AIDS-KS cells (27). To determine whether OM, LIF, and IL-6 share the activity as an AIDS-KS cell mitogen, AIDS-KS growth assays for three isolates of KS cells (KS-USC22, KS10B, and KS3 cells) were performed using recombinant human OM, LIF, and IL-6 at various concentrations. As shown in Table I, growth stimulation of AIDS-KS cells was exclusively observed in response to OM and not to LIF or IL-6. None of the three different isolates used in this study exhibited significant growth augmentation by LIF or by IL-6. In addition, no growth-inducing effects by IL-6 were observed on 14 isolates derived from different patients (Table II). A possible explanation for the OM-specific biological activity for AIDS-KS cell growth may be the existence of an OM-specific receptor and the absence of LIF and IL-6 receptors on AIDS-KS cells. To examine this possibility, binding experiments on three isolates of KS cells using ^{35}S-labeled LIF or IL-6 performed (Fig. 3). Compared with binding of ^{35}S-labeled OM, neither ^{35}S-labeled LIF nor IL-6 bound to AIDS-KS cells, a finding that shows the lack of LIF and IL-6 receptors on AIDS-KS cells. Thus, the specificity in binding probably contributes to the OM-specific growth stimulating effect on AIDS-KS cells.

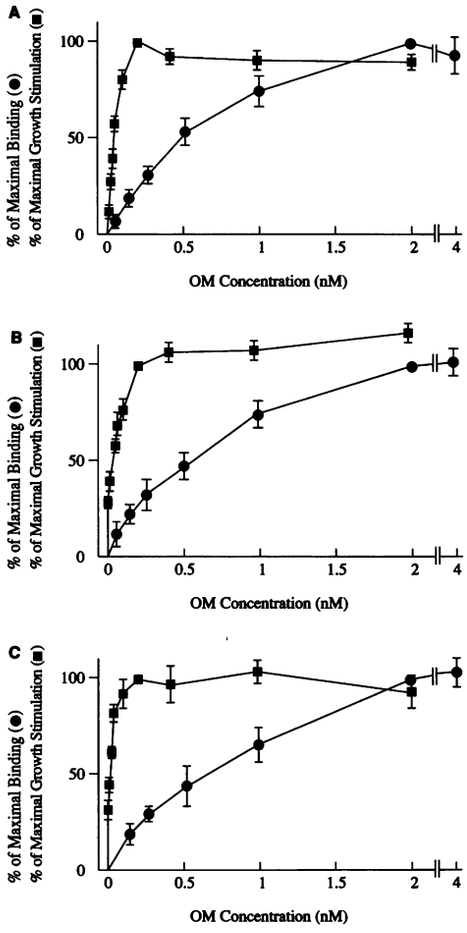

Figure 2. Comparison of biological activity and binding of ^{35}S-labeled OM to AIDS-KS cells. (*A*) AIDS-KS–USC22, (*B*) -KS10B, and (*C*) -KS3 cells in duplicate were tested for responsiveness to growth stimulation by ^{35}S-labeled OM and for binding of ^{35}S-labeled OM, as described in Methods, and then compared with the percentage of the maximal biological response to ^{35}S-labeled OM and the maximal level of ^{35}S-labeled OM binding. Each point represents the mean±SD (n = 4) of duplicate determinations from two separate experiments. 100% of maximal growth stimulation was set as the level of growth stimulation obtained at 0.2 nM of ^{35}S-labeled OM. 100% binding was set as the level of ^{35}S-labeled OM binding observed at 2 nM of ^{35}S-labeled OM.

Table I. Effects of OM, LIF, and IL-6 on Growth of AIDS-KS Cells

Cytokines	Numbers of AIDS-KS cells		
	KS3	KS10B	KS-USC22
Medium control	7,552±364	6,430±722	5,184±512
rhOM (10 ng/ml)	30.495±2,494	22,050±1,960	29,000±2,060
rhIL-6 (ng/ml)			
3.2	9,670±312	6,456±416	4,086±294
6.2	9,492±540	7,182±286	4,722±66
12.5	7,146±586	7,944±526	4,860±852
25.0	7,902±112	6,606±104	4,185±462
50.0	7,794±194	6,840±146	4,788±132
rhLIF (ng/ml)			
3.2	4,692±264	5,652±392	5,538±90
6.2	6,618±340	7,560±318	4,734±498
12.5	7,548±512	5,688±108	5,316±72
25.0	8,718±406	5,424±176	5,532±600
50.0	6,018±560	5,400±430	5,598±54

3×10^3 AIDS-KS cells/well were cultured in 24-well plates in medium (RPMI 1640 supplemented with 15% FBS), with or without indicated cytokines. A Coulter particle counter was used to estimate cell growth at 6 d of culture. Data are expressed as mean±SD of triplicate determinations from two separate experiments (n = 6).

Table II. Lack of Effects of IL-6 on Growth of 14 Different Isolates of AIDS-KS Cells

Isolates	Origins	Number of AIDS-KS cells			
		Medium control	rhIL-6		rhOM (10 ng/ml)
			10 ng/ml	50 ng/ml	
KS1	PF	4,368±720	4,623±261	4,920±453	23,784±933
KS2	PF	6,924±720	6,384±363	6,832±465	28,896±789
KS3	LG	7,248±423	7,320±466	7,452±194	30,660±2,568
KS4	PF	4,896±231	3,936±264	4,800±369	25,632±1,662
KS5	PF	5,868±171	6,204±420	5,568±480	30,324±1,497
KS6	PF	3,660±300	3,720±267	3,600±126	29,556±1,245
KS7	PF	4,800±807	4,704±282	4,896±141	28,808±1,203
KS8	LG	4,344±714	4,512±660	4,608±711	27,960±1,212
KS9	PF	5,604±423	5,316±438	5,400±477	29,196±2,475
KS10B	OM	6,324±689	7,032±552	6,876±263	23,064±2,087
KS11	PF	4,680±363	4,212±528	4,488±600	30,228±1,743
KS12	PF	5,088±405	5,172±573	5,172±318	28,464±576
KS13	LG	5,604±750	5,544±552	5,316±525	31,752±1,428
KS-USC22	PF	5,292±564	4,884±498	4,992±678	29,472±2,065

3×10^3 AIDS-KS cells/well were cultured in 24-well plates in medium (RPMI 1640, 15% FBS), with or without indicated cytokines. A Coulter particle counter was used to estimate cell growth at 6 d of culture. Data are expressed as mean±SD of triplicate determinations from two separate experiments. LG, lung KS; OM, oral mucosa KS; PF, pleural effusion of lung KS.

AIDS-KS cells express the OM-specific receptor but not the LIF/OM receptor. Recent studies show that LIF and OM bind with a high affinity to the same receptor, LIF/OM receptor, which consists of the LIF receptor α-subunit and gp130 (24). We asked whether AIDS-KS cells express the LIF/OM receptor or utilize the OM-specific receptor as the OM binding site. To investigate the existence of an LIF/OM receptor and the sharing of this receptor by LIF and OM on AIDS-KS cells, competition experiments for ^{35}S-labeled OM binding to KS-USC22, KS10B, and KS3 cells by unlabeled OM, IL-6, and LIF were performed (Fig. 4). Binding of ^{35}S-labeled OM was displaced by unlabeled OM, which again indicated the presence of a specific receptor for OM and the absence of the LIF/OM receptor on AIDS-KS cells. As expected from the lack of any detectable binding of ^{35}S-labeled LIF and IL-6 to AIDS-KS cells (Fig. 3), no significant effect of unlabeled LIF or IL-6 on ^{35}S-labeled OM binding was observed on these isolates of KS cells. Thus, OM apparently does not share receptor sites with LIF on AIDS-KS cells, indicating that KS cells do not express the LIF/OM receptor. AIDS-KS cells carry an OM-specific receptor that is unable to bind LIF or IL-6, and through which OM exerts growth-stimulating effects on AIDS-KS cells.

Anti-gp130 antibody can completely abrogate transduction of the OM-mediated growth-stimulating signal and OM binding to AIDS-KS cells. Several groups of workers reported that gp130, a signal transducer and affinity converter for both LIF and IL-6, is capable of binding OM with low affinity (24, 25, 32, 33). To determine if gp130 mediates the biological response of OM through the OM-specific receptor on AIDS-KS cells, KS growth assays were carried out in the presence of anti-human gp130 antibodies. As shown in Fig. 5, anti-gp130 antibodies dose-dependently prevented the AIDS-KS growth stimulation caused by OM. Complete inhibition was achieved by 1 μg/ml of anti-gp130 antibodies. Control mouse antibody did not abolish the growth stimulated by OM. Inhibition of OM-induced KS cell growth by anti-gp130 antibodies was observed in experiments performed with three isolates of KS cells (KS-USC22, KS10B, and KS3 cells). These results indicate that gp130 is involved in transducing the OM-mediated growth-stimulating signal to AIDS-KS cells and is even a component of the OM-specific receptor on KS cells. To elucidate the role of gp130 in OM binding for AIDS-KS cells, we examined the potential of anti-gp130 antibody to block the binding of ^{35}S-labeled OM on these isolates of AIDS-KS cells (Fig. 6). Binding of ^{35}S-labeled OM to AIDS-KS cells was blocked by 10 μg/ml of anti-gp130 antibodies but was unaffected by control antibody. Thus, the growth inhibition of AIDS-KS cells by anti-gp130 antibodies is apparently associated with the blocking of OM binding to gp130 on these cells. Therefore, it seems likely that the OM-specific receptor on AIDS-KS cells includes gp130 and that gp130 can function as an OM binding subunit.

Cross-linking of ^{35}S-labeled OM to AIDS-KS cells. To chemically identify the molecular species of the OM-specific receptor on AIDS-KS cells, we performed cross-linking studies with ^{35}S-labeled OM, using DSS, a bifunctional cross-linking reagent (Fig. 7). SDS-PAGE analysis of OM binding proteins on AIDS-KS cells displayed one major species which migrated as a broad band of 180–220 kd. In addition, another cross-linked species with an apparent molecular mass of 50 kD was observed. The degree of cross-linking of ^{35}S-labeled OM to these species was greatly reduced in the presence of a 100-fold excess of unlabeled OM, but not with LIF or IL-6, thereby confirming the specificity of the cross-linking reaction and suggesting that AIDS-KS cells carry the OM-specific receptor not shared with LIF or IL-6. The cross-linking of ^{35}S-labeled OM to AIDS-KS cells was also completely abolished by anti-gp130 antibodies, but not by control mouse antibody, a finding that strongly supports the notion that the 180–220 kD species includes gp130 cross-linked by ^{35}S-labeled OM. These results agree with the results of the

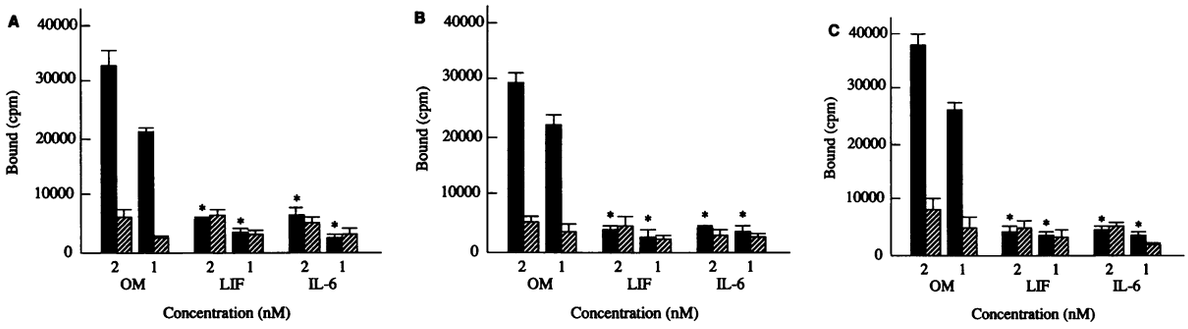

Figure 3. Lack of binding properties of ^{35}S-labeled LIF and IL-6 to AIDS-KS cells. (*A*) AIDS-KS–USC22, (*B*) -KS10B, and (*C*) -KS3 cells (2.5 × 10^4 cells) in duplicate wells were incubated with 1 nM and 2 nM ^{35}S-labeled recombinant human OM, LIF, or IL-6 alone (*closed bar*), or plus 100-fold excess of respective unlabeled recombinant cytokines (*hatched bar*) for 3 h at room temperature. Data represent average cell-bound counts ±SD of duplicates from four separate experiments (n = 8). The asterisk (*) indicates conditions in which $P < 0.005$ as compared with the total binding at the corresponding concentration of ^{35}S-labeled OM.

binding assays and those of the in vitro growth assays for AIDS-KS cells. This species seems to be an OM-specific receptor complex including OM, gp130, and/or another unknown subunit (putative β-subunit of the OM-specific receptor). Considering the relative molecular weight of this species, it is unlikely that this species includes two molecules of gp130. While the significance of the 50-kD species is not clear, similar observations were noted for other OM responsive cells (29). This band might represent another subunit of the OM-specific receptor.

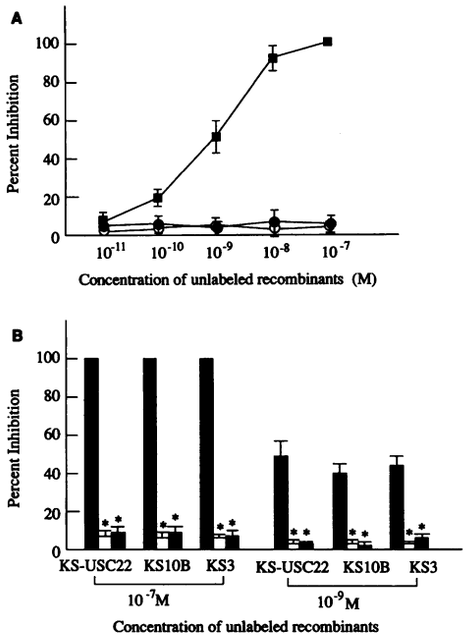

Figure 4. Effects of unlabeled OM, LIF, or IL-6 on ^{35}S-labeled OM binding on AIDS-KS cells. *(A)* Binding of a constant amount (5 × 10^{-10} M) of ^{35}S-labeled OM was determined in the presence of several dilutions of unlabeled recombinant OM *(closed squares)*, LIF *(closed circles)* or IL-6 *(open circles)* on AIDS-KS cells (2.5 × 10^4 cells) in duplicate wells, as described in Methods. The data were plotted after subtraction of nonspecific binding and conversion to percent inhibition. 100% inhibition was set as the level of binding observed in the presence of 1 × 10^{-7} M unlabeled OM. Each point represents the mean±SD of duplicate determinations from four separate experiments (n = 8). *(B)* The data obtained in similar experiments performed with AIDS-KS10B and -KS3 are shown. Values are the means±SD of duplicate determinations from three independent experiments (n = 6). *Closed bars,* with unlabeled OM; *open bars,* with unlabeled LIF; *hatched bars,* with unlabeled IL-6. The asterisk (*) indicates conditions in which $P < 0.005$ as compared with the level of binding observed in the presence of the corresponding concentration of unlabeled OM.

AIDS-KS cells express gp130 mRNA, but not mRNA for LIF receptor α-subunit or IL-6 receptor α-subunit. The expression of specific mRNA was demonstrated by the appearance of PCR amplification products (Fig. 8). PCR amplification, using ds cDNA for four isolates of AIDS-KS cells (KS-USC22, KS10B, KS3, and KS14), revealed considerable amounts of expression of gp130 mRNA and only scanty amounts of PCR products for the LIF receptor α-subunit or the IL-6 receptor α-subunit were observed. Similar results were also obtained with Northern hybridization (data not shown). These findings mean that neither the LIF/OM receptor nor the IL-6 receptor is expressed on AIDS-KS cells. In addition, the results agree with data showing the lack of any binding of LIF and IL-6, since these α-subunits are reported to serve as binding subunits in their respective receptors.

Discussion

Thoma et al. (34) suggested the existence of two different types of the high affinity receptor for OM. Type I receptor (LIF/OM receptor) binds both OM and LIF, which appears to consist of two subunits: the LIF receptor α-subunit and gp130 (24). The existence of the LIF/OM receptor may explain overlapping biological responses of LIF and OM. Type II receptor is an OM-specific receptor, the existence of which may agree with findings that several biological responses are exclusively induced by OM but not by LIF (16, 17), although the biological relevance and molecular structure of the OM-specific receptor have not been determined. To investigate whether AIDS-KS cells carry the OM/LIF receptor of the OM-specific receptor, or both, we characterized OM receptor binding sites on AIDS-KS cells and found that AIDS-KS cells express the OM-specific receptor, but not the OM/LIF receptor, as the functional high affinity binding site for OM: *(a)* OM, but not LIF, can elicit a growth-stimulating response for AIDS-KS cells (Table I); *(b)* AIDS-KS cells are capable of binding OM, but not LIF, with high and low affinity (Figs. 1 and 3); *(c)* in competition studies,

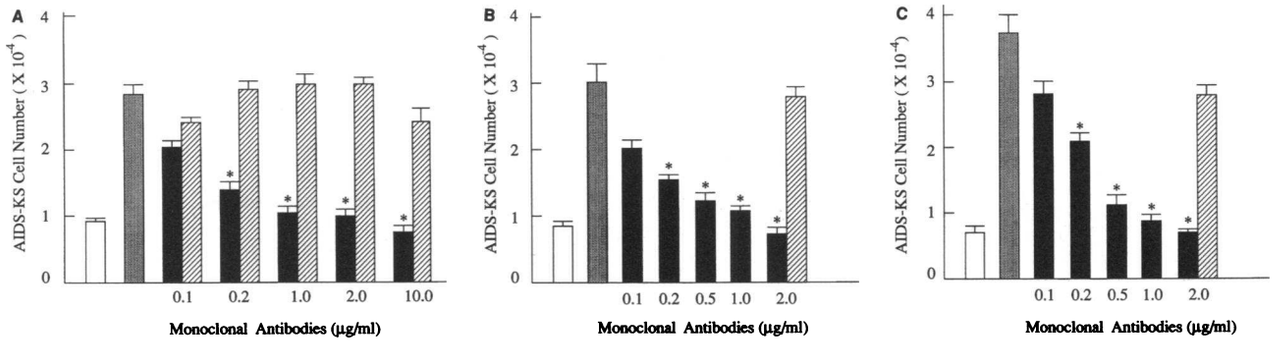

Figure 5. Inhibition of growth-stimulating effects of OM by anti-gp130 antibodies. *(A)* AIDS-KS–USC22, *(B)*-KS10B, and *(C)*-KS3 cells (3×10^3 cells) in triplicate wells were incubated on 24-well plates for 6 d in medium (RPMI 1640, 15% FBS) alone, or supplemented with 10 ng/ml of OM, with and without various amounts of anti-gp130 monoclonal antibodies or control mouse monoclonal antibody. A mixture of equal amounts of anti-gp130 monoclonal antibodies GPX7, GPX22, and GPZ35 was used. Values are means ±SD of triplicate determinations of two independent experiments ($n = 6$). *Open bar,* medium alone; *dotted bar,* with 10 ng/ml OM; *closed bar,* with 10 ng/ml OM plus anti-gp130 antibodies; *hatched bar,* with 10 ng/ml OM plus control mouse antibody. The asterisk (*) indicates significant decrease as compared with KS cell number in the presence of 10 ng/ml OM alone ($P < 0.01$).

OM binding and cross-linking to AIDS-KS cells are not displaced by excess LIF (Figs. 4 and 7); and *(d)* AIDS-KS cells express considerable amounts of the transcript for gp130, whereas the transcript of the LIF receptor α-subunit was not observed (Fig. 8). Thus, the results of this study provide evidence that OM-induced growth stimulation for AIDS-KS cells correlates with the expression of the OM-specific receptor but not with the LIF/OM receptor.

gp130, a membrane glycoprotein, was originally identified in a myeloid leukemia cell line as one component of IL-6 receptor (32, 33). Several studies showed that gp130 is shared with IL-6 and LIF as a common signal transducer and affinity converter of their receptor, although gp130 alone cannot bind either LIF or IL-6 (24, 32). LIF and IL-6 also have individual binding subunits (α-subunits), which form low affinity receptors. Association of gp130 to the individual binding subunits is required for high affinity binding to the respective cytokines and for signaling of biological activities. Recent studies indicate that gp130 can bind OM with low affinity (24, 25). In addition, the LIF/OM receptor consists of gp130 and LIF receptor α-subunits (24). However, it has not been determined if the OM-specific receptor includes gp130 as an essential component for OM binding and/or signaling. Here, we found that *(a)* anti-gp130 antibodies can block the growth-stimulating effect of OM on AIDS-KS cells (Fig. 5); *(b)* the binding of OM to AIDS-KS cells is abolished by anti-gp130 antibodies (Fig. 6); *(c)* OM binding species detected in cross-linking experiments are blocked by anti-gp130 antibodies (Fig. 7); and *(d)* the transcript for gp130 is highly expressed in AIDS-KS cells, as observed in PCR amplification (Fig. 8). Because we also obtained evidence for expression of the OM-specific receptor on AIDS-KS cells and its correlation with OM-specific growth induction for KS cells, gp130 seems an essential component of the OM-specific receptor on KS cells for OM binding and OM-induced growth. However, it remains to be determined whether OM binding to gp130 requires accessory factor(s) and whether gp130 alone can transduce OM-elicited growth-stimulating signals to AIDS-KS cells. Liu et al. suggested that gp130 alone is not sufficient to transduce OM-elicited growth modulation signals and that an additional component(s) is required for formation of the functional high affinity receptor for OM, determined by using gp130-transfected cells (35). It is likely that the OM-specific high affinity receptor on AIDS-KS cells consists of gp130, which can bind OM directly, and also the putative OM receptor β-subunit, which is essential for high affinity binding and signal transduction on KS cells.

Miles et al. reported that AIDS-N521J KS cells (or KS2) expressed mRNA for IL-6 receptor α-subunit. They also showed that the antisense oligonucleotide for IL-6 decreased cellular proliferation of AIDS-KS cells (27). Conversely, we found here that *(a)* addition of IL-6 to cultures of AIDS-KS cells (in growth assays with 14 different isolates, including N521J cells) has little effect on growth (Tables I and II); *(b)* IL-6 cannot bind to AIDS-KS cells in binding assays (Fig. 3); and *(c)* PCR amplification and Northern blot analysis revealed that the IL-6 receptor α-subunit was not expressed in four representative KS cell isolates (Fig. 8). Moreover, we observed that anti-IL-6 antibody had no effect on proliferation of AIDS-KS cell (data not shown), although KS cells produce and secrete IL-6 (12). If expression of the IL-6 receptor α-subunit can be induced on AIDS-KS cells, the disparities observed between these studies can be explained by the differential expression of the IL-6 receptor α-subunit. In a previous study (27), N521J cells, which did not proliferate in response to IL-6 in our culture conditions, may have been exposed to a stimulator(s) for expression of the IL-6 receptor α-subunit. However, we could not determine whether possible culture conditions or stimulators induced the IL-6 receptor α-subunit in growth assays performed with 14 different isolates, nor could we develop an AIDS-KS cell isolate expressing the IL-6 receptor. Taking all these data into consideration, it seems unlikely that the growth of AIDS-KS cells is mediated by IL-6.

We obtained evidence that the lack of IL-6- and LIF-induced growth stimulation for KS cells can be explained by the absence of the IL-6 receptor and the LIF/OM receptor on these cells. For AIDS-KS cells, the differences in biological responses among OM, LIF, and IL-6 probably arise from specificity in their binding. A recent study showed that OM induces tyrosine protein phosphorylation and MAP kinase (mitogen activated protein kinase) activation in AIDS-KS cells, while neither LIF nor IL-6 activates this signal transduction pathway (36). These observations support our findings that AIDS-KS cells express

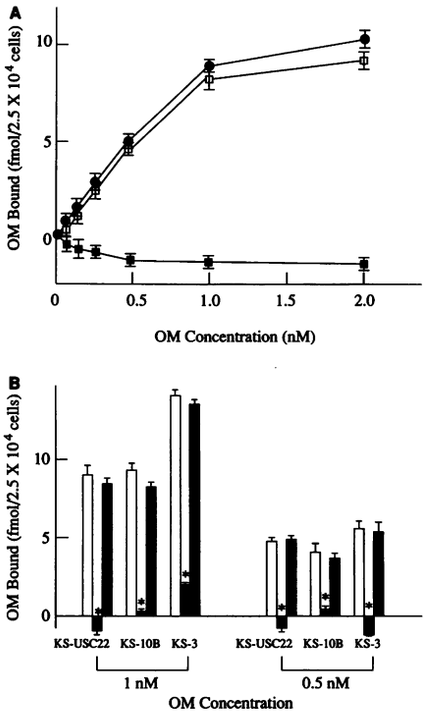

Figure 6. Blocking of [^{35}S] OM binding to AIDS-KS cells by anti-gp130 antibodies. *(A)* AIDS-KS–USC22 cells (2.5×10^4 cells) in duplicate were incubated with binding buffer alone *(closed circles)*, or supplemented with 10 μg/ml anti-gp130 antibodies *(closed squares)* or 10 μg/ml control mouse antibody *(open squares)* for 60 min at room temperature. A mixture of anti-gp130 monoclonal antibodies GPX7, GPX22, and GPZ35 was used. Then various amounts of [^{35}S] OM were added, with or without 100-fold excess of unlabeled OM, and the cells were incubated for another 3 h at room temperature. Specific binding was expressed as the difference between total and nonspecific binding. Values are means±SD of duplicate determinations of three independent experiments ($n = 6$). *(B)* Similar experiments were performed with AIDS-KS10B and -KS3 cells. Data obtained at 0.5 nM and 1.0 nM of [^{35}S] OM are shown. Values are means±SD of duplicate determinations from two separate experiments ($n = 4$). *Open bars,* binding buffer alone; *hatched bars,* with 10 μg/ml control mouse antibody; *closed bars;* with 10 μg/ml anti-gp130 antibodies. The asterisk (*) indicates a significant decrease as compared with OM binding in the presence of OM alone ($P < 0.005$).

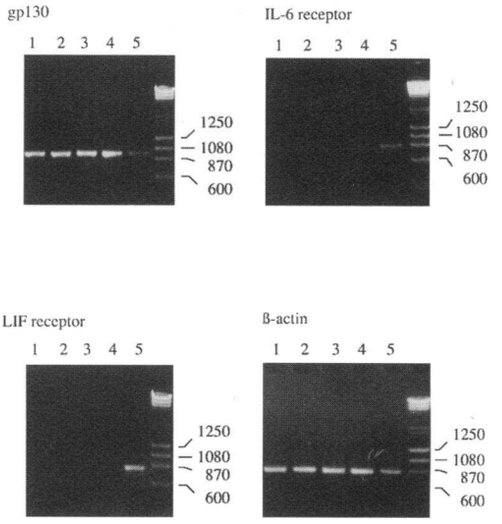

Figure 8. Detection of transcripts for gp130, LIF receptor α-subunit, and IL-6 receptor α-subunit in AIDS-KS–USC22, -KS3, and -KS10B cells. PCR amplification: equal amounts of double strand cDNA generated from poly $(A)^+$ RNA of four isolates of AIDS-KS cells were subjected to gp130-specific, LIF receptor α-subunit–specific, and IL-6 receptor α-subunit–specific PCR amplifications using paired primers, respectively. RNA integrity and the efficiency of cDNA synthesis were confirmed by PCR amplification, using a human β-actin–specific primer (Clontech, Palo Alto, CA). PCR amplification was performed under the following condition: 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min; 1 cycle at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 10 min. The amplification products were electrophoresed on 1.2% agarose gels. The primers for OM, LIF receptor, IL-6 receptor, and β-actin directed the amplification product corresponding to 960 bp, 791 bp, 826 bp, and 838 bp, respectively. Positions of size standards are shown. To check the efficiency of PCR, approximately 100 attomoles (60 pg) of the ds cDNA fragments encoding gp130, LIF receptor α-subunit, IL-6 receptor α-subunit, and β-actin were included as templates in PCR amplifications, using the corresponding primers (positive controls). These fragments, except for β-actin, were prepared by excising from pCRII-gp130, pCRII-LIFRec and pCRII-IL6Rec vectors as described in Methods. The cDNA fragment for β-actin was purchased from Clontech. Lane *1,* AIDS-KS–USC 22; lane *2,* AIDS-KS 3; lane *3* AIDS-KS 10B; lane *4,* AIDS-KS 14; lane *5,* positive controls (human liver for gp130 and LIF receptor; U937 for IL-6 receptor).

neither the IL-6 nor LIF/OM receptor. The lack of binding of these cytokines to KS cells can explain failure to activate postreceptor pathways for signal transduction. OM was reported to be a major growth factor for rabbit vascular smooth muscle cells (SMC), while LIF or IL-6 did not affect SMC DNA synthesis (17). Because the origin of KS cells was suggested to be a vascular SMC progenitor (12), the similarity in growth response between AIDS-KS cells and SMCs is intriguing.

We found that anti-gp 130 antibodies can completely block OM-induced AIDS-KS cell growth (Fig. 5). KS has been reported to be a proliferative disease induced by several cytokines, which are produced and secreted by AIDS-KS cells (6–8). A strategy for producing human monoclonal antibodies in mice has been described (37), and such mice should permit the development of human monoclonal antibodies with therapeutic potential. Anti-gp 130 antibodies may prove to be a potential therapeutic drug for KS.

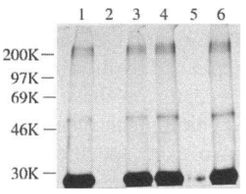

Figure 7. Chemical cross-linking of [^{35}S] OM to binding sites on AIDS-KS–USC22, -KS3, and -KS10B cells. AIDS-KS–USC 22 cells (2.5×10^4 cells) were incubated with 1 nM [^{35}S] OM alone (lane *1*), or supplemented with 100-fold excess of unlabeled OM (lane *2*), LIF (lane *3*), IL-6 (lane *3*), IL-6 (lane *4*), 10 μg/ml anti-gp130 monoclonal antibodies (lane *5*) or 10 μg/ml control monoclonal antibody (lane *6*) for 3 h at room temperature. Chemical cross-linking of [^{35}S] OM to AIDS-KS cells is described in Methods. Positions of molecular weight markers are indicated on the right.

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