*Brief Definitive Report*

# Apolipoprotein E (ApoE), a Novel Heparin-binding Protein Inhibits the Development of Kaposi's Sarcoma-like Lesions in BALB/c *nu/nu* Mice

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## Summary

Recombinant apolipoprotein E-3 (ApoE-3), expressed in *Escherichia coli*, was purified and used in an in vitro and an in vivo model system for acquired immunodeficiency syndrome–associated Kaposi's sarcoma (AIDS-KS). This protein blocked cell proliferation and chemotaxis of AIDS-KS cells in response to activated lymphocyte conditioned medium (AL-CM) and oncostatin M (OSM). ApoE-3 also inhibited the formation of neoangiogenic lesions induced in BALB/c *nu/nu* mice by AIDS-KS cells. These findings represent a novel and potentially less toxic therapeutic approach for the treatment of AIDS-KS.

We previously developed an in vitro and an in vivo model system (1, 2) to study the pathogenesis of AIDS-associated Kaposi's sarcoma (AIDS-KS) (3–7). Recently, we reported on a novel approach for the treatment of AIDS-KS using a bacteria-derived sulfated polysaccharide-peptidoglycan termed DS-4152 (8–11). This observation stimulated our interest in understanding the role of other sulfated glycans including heparin/heparan sulfate proteoglycans (HSPG) (12), and the molecules that interact with HSPG in the regulation of AIDS-KS cell growth, chemotaxis, and neoangiogenesis.

Several plasma proteins including platelet factor 4 (13), osteonectin/secreted protein acidic and rich in cysteine (14), and thrombospondin (15–17) have antiangiogenic properties in vitro and in vivo. Platelet factor 4 (13) and thrombospondin (15–17) have antiangiogenic properties in vitro and in vivo, and are strong heparin-binding proteins as well. Another protein that is ubiquitously expressed and has strong affinity for heparin and HSPG is Apolipoprotein E (ApoE) (18). Recently, we demonstrated that recombinant ApoE-3 (19) specifically inhibited the in vitro proliferation of bovine endothelial and human smooth muscle cells, as well as several human tumor cell lines (20). Through receptor binding studies we demonstrated that ApoE-3 competed with basic fibroblast growth factor for binding to the low affinity fibroblast growth factor receptor (20).

In the present report we investigated further whether ApoE-3 could function as an antiangiogenic molecule in our in vitro and in vivo AIDS-KS model system. The findings presented suggest a novel and potentially less toxic therapeutic approach for the treatment of AIDS-KS (3, 4, 6, 7, 21).

## Materials and Methods

*Reagents.* Human recombinant ApoE-3 isoform was expressed in *Escherichia coli* and purified as previously described (19). Purified recombinant human ApoE-3 was first lyophilized from a solution of 1 mg of ApoE-3/ml containing 2 mM sodium bicarbonate and 1 mM cysteine. The lyophilized ApoE-3 was stored at −70°C; under these conditions the recombinant ApoE-3 is stable for years. ApoE-3 was reconstituted in sterile distilled water at concentrations of 1–10 mg/ml. To this solution, 10% (vol/vol) of 10× Dulbecco's PBS (D-PBS) (GIBCO BRL, Gaithersburg, MD) was added. The ApoE-3 solutions were used immediately, or stored in small aliquots at −20°C and used within 2–3 wk. Activated lymphocyte conditioned media (AL-CM) was prepared from PHA-activated PBMC. Peripheral blood was obtained from normal volunteers at the National Institutes of Health Blood Bank. PBMC were isolated using lymphocyte separation medium (Organon Teknika Corp., Durham, NC). This medium allows the separation of granulocytes and red blood cells from the mononuclear cells, e.g., lymphocytes, monocytes, dendritic cells, etc., by density centrifugation. Isolated PBMC were cultured in RPMI 1640 (GIBCO BRL) containing 10% FCS (GIBCO BRL) and 2 μg/ml of Bacto-phytohemagglutinin-P (Difco Laboratories, Detroit, MI), 2 × $10^6$ cells/ml in RPMI 1640 containing 10% FCS and 20 U/ml of IL-2 (Boehringer Mannheim Corp., Indianapolis, IN). After 72 h, the AL-CM was collected, filtered through a 0.45-micron filter (Corning Glass Co., Corning, NY), aliquoted, and stored at −20°C. AL-CM that stimulated a

threefold increase in AIDS-KS cell number after 7 d was used in these studies. Oncostatin M (OSM) (22) levels were determined in the AL-CM using the OSM Quantikine Immunoassay kit (R & D Systems, Minneapolis, MN). The AL-CM prepared as above contained 20–40 pg/ml of OSM (22). Recombinant human OSM (22) was obtained from PeproTech (Minneapolis, MN).

*Cells.* AIDS-KS cells were isolated and cultured as previously described (1). The AIDS-KS cell strain RW248 was isolated from the pleural effusion of a patient with AIDS-KS. The RW248 AIDS-KS cell strain has a normal karyotype, and the cell phenotype and growth characteristics in vitro and in vivo are similar to the other AIDS-KS cell strains previously described (1, 2).

*Thymidine Incorporation into DNA.* DNA synthesis was determined as follows: the KS cell strain, RW248, was grown to confluence, at which time the medium was replaced with fresh RPMI 1640 (GIBCO BRL) containing 0.5% FCS (GIBCO BRL) and cultured for 72 h to growth-arrest the cells. The cells were trypsinized, plated at a density of $2 \times 10^4$ cells/well in 24-well tissue culture plates (Becton Dickinson & Co., Lincoln Park, NJ), and cultured in RPMI 1640, 1% FCS with either 20% AL-CM medium or OSM (22) 30 ng/ml, and increasing concentrations of native ApoE-3 (19). Each concentration of ApoE-3 was assayed in triplicate. After 24 h, the cells were pulsed with 1 $\mu$Ci/ml of [methyl-$^3$H]thymidine (New England Nuclear [NEN], Boston, MA) (1 Ci = 37 GBq) for 12 h. After 12 h triplicate wells were washed twice with cold PBS, fixed at room temperature with 1 ml of methanol/acetic acid (3:1) for 1 h, washed twice with 80% ethanol, and allowed to air dry. To each well, 300 $\mu$l of 0.25% trypsin (GIBCO BRL) wash was added and incubated at 37°C for 30 min, and then 100 $\mu$l of 10% sodium dodecyl sulfate was added and the contents in each well were dissolved in 10 ml of Aquasol (NEN) for scintillation counting.

*Cell Proliferation Assay.* For cell proliferation assays nongrowth-arrested cells, $10^4$ cells/well were seeded in triplicate in 96-well flat bottomed plates (Becton Dickinson & Co.) that contained the required dilutions of native ApoE-3 (19), heat inactivated ApoE-3 (19), or buffer in RPMI 1640 containing 1% FCS. After 3 d the cell density was determined using the protocol outlined in the cell proliferation assay kit (Promega Corp., Madison, WI). This assay is based on the cellular conversion of tetrazolum salt into a blue formazan product that can then be detected by measuring absorbance at 570 nm.

*Cell Migration Assay.* Chemotaxis of the RW248 AIDS-KS cells was performed in a modified Boyden chamber assay. 8-micron pore polyvinylpyrrolidine-free nucleopore polycarbonate filters (Neuro Probe, Inc., Cabin John, MD) were coated with gelatin (0.1 mg/ml in 0.1% acetic acid). Subconfluent AIDS-KS cells, RW248, were harvested the first day after passage by brief treatment with 0.05% trypsin and 0.02% EDTA. The cells were reequilibrated for 2 h at room temperature in RPMI 1640 containing 10% FCS (Biofluids, Rockville, MD) and 20% AL-CM. Chemoattractants OSM (22) or AL-CM and cells at $0.5 \times 10^6$ cells/ml were resuspended in RPMI 1640 containing 0.1% BSA (Sigma Chemical Co., St. Louis, MO). 28 $\mu$l of each chemoattractant were loaded into the lower chamber wells. 56 $\mu$l of cells ± ApoE-3 (19) (at the indicated concentrations) were loaded in the upper chamber wells. Migration was performed at 37°C for 3.5 h. The filters were fixed, stained, and quantified microscopically by counting cells in three separate areas of each stained well. The nonstimulated migration determined at each ApoE-3 (19) concentration was subtracted from stimulated motility and is expressed as percentage of the respective controls. The values are mean of triplicate determinations and the bars indicate standard deviations, $n = 3$.

*Angiogenesis In Vivo.* $2 \times 10^6$ AIDS-KS cells, RW248 passage 6-8, were transplanted subcutaneously on day 0 on the backs of male BALB/c *nu/nu* athymic mice. The mice were administered PBS, ApoE-3 (19) 0.2 mg, ApoE-3 (19) 0.4 mg, or ApoE-3 (19) 0.8 mg i.v. on days 0–4. On day 6 the animals were killed, and the KS-like lesions were measured along with other macroscopic characteristics. The values are representative of four in vivo experiments representing the mean and the error bars of the standard deviations. Statistical analysis was performed using the two-tailed Student's $t$ test.

## Results and Discussion

*Inhibition of DNA Synthesis and Cell Proliferation by ApoE-3.* In a dose-dependent manner, both cell proliferation (Fig. 1 *A*) and DNA synthesis (Fig. 1, *B* and *C*) were inhibited when ApoE-3 (19) was added to growth media containing either AL-CM (Fig. 1 *B*) or OSM (22) (Fig. 1, *A* and *C*). OSM (22) a cytokine secreted into the conditioned medium of activated lymphocytes, is a member of the neuropoietic cytokine family and one of the most effective exogenous growth factors for the AIDS-KS cells (23–25). This inhibitory effect was abolished if the ApoE-3 (19) was heat inactivated, suggesting that the inhibitory effect requires the native conformation of ApoE-3 (Fig. 1 *A*). ApoE-3 (19) blocked the release from growth arrest when AL-CM (Fig. 1 *B*) or OSM (Fig. 1 *C*) were added to growth arrested AIDS-KS cells. Release from growth arrest is required to recruit new endothelial cells for angiogenesis (26, 27). The concentrations of ApoE-3, required to inhibit DNA synthesis by 50% after growth arrest, were 0.07 $\mu$M in the presence of 20% AL-CM (Fig. 1 *B*), and 0.7 $\mu$M in the presence of recombinant OSM (22), 50 ng/ml (Fig. 1 *C*). The stronger inhibitory activity of ApoE-3 in the presence of AL-CM may result from the much lower levels of OSM in AL-CM than the exogenous OSM used in these experiments. These data suggest that ApoE-3 is a potential antiangiogenic molecule that functions by inhibiting growth-arrested cells e.g., vascular endothelium or the AIDS-KS cells from responding to growth factors. In addition, ApoE-3 inhibits AIDS-KS cell proliferation even if cells are not growth arrested by serum starvation (Fig. 1 *A*), suggesting more than one mechanism of action.

*Inhibition of Migration by ApoE-3.* Migratory properties of the AIDS-KS cells (1) were also inhibited by ApoE-3 (Fig. 2). Migration of endothelial cells in response to chemotactic stimuli is a key aspect of angiogenesis. Previous work (28–30) in our laboratory demonstrated that AL-CM contains a number of growth factors that may influence angiogenesis including IL-1, IL-6, TNF-$\alpha$, and OSM that may also function as chemoattractants for the AIDS-KS cells. These factors in vivo could contribute to the invasiveness and multifocal lesions characteristic of the AIDS-KS lesions found in patients.

The migration of the AIDS-KS cells (1) to the chemoattractants AL-CM and OSM (22) was tested using a modified Boyden chamber assay. AL-CM and OSM stimulated by two- to threefold the migration of AIDS-KS cells from the upper chamber to the lower chamber. The optimal concentration of AL-CM and OSM that stimulated cell migration also maximally stimulated the growth of the AIDS-KS cells. ApoE-3

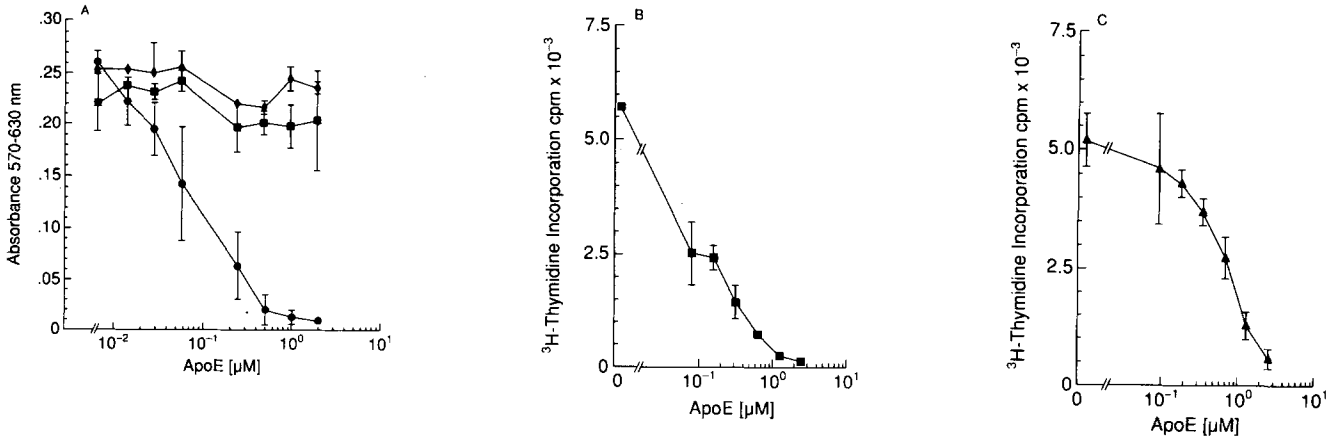


**Figure 1.** Assays for DNA synthesis and cell proliferation of the AIDS-KS cells in the presence of ApoE-3 and growth factors. For cell proliferation assays (*A*) nongrowth-arrested cells, $10^4$ cells/well were seeded in triplicate in 96-well flat-bottomed plates (Falcon Labware, Franklin Lakes, NJ) that contained the required dilutions of native ApoE-3 (●), heat-inactivated ApoE-3 (t), or buffer (■) in RPMI 1640 containing 1% FCS. After 3 d the cell density was determined using the protocol outlined in the cell proliferation assay kit (Promega Corp., Madison, WI). This assay is based on the cellular conversion of tetrazolum salt into a blue formazan product that is detected by measuring absorbance at 570 nm. DNA synthesis (*B*) and (*C*) was determined as follows: the KS cell strain, RW248 was grown to confluence, at which time the medium was replaced with fresh RPMI 1640 (GIBCO BRL) containing 0.5% FCS (GIBCO BRL) and cultured for 72 h to growth-arrest the cells. The cells were trypsinized, plated at a density of $2 \times 10^4$ cells/well in 24-well tissue culture plates (Falcon Labware), and cultured in RPMI 1640, 1% FCS, with either 20% AL-CM medium (■) (12) (*B*) or OSM 30 ng/ml (▲) (PreproTech) (*C*), and increasing concentrations of native ApoE-3. Each concentration of ApoE-3 was assayed in triplicate. After 24 h the cells were pulsed with 1 μCi/ml of [methyl-$^3$H]thymidine (NEN) (1 Ci = 37 GBq) for 12 h. After 12 h, triplicate wells were washed twice with cold PBS, fixed at room temperature with 1 ml of methanol/acetic acid (3:1) for 1 h, washed twice with 80% ethanol, and allowed to air dry. To each well 300 μl of 0.25% trypsin (GIBCO BRL) wash was added and incubated at 37°C for 30 min, then 100 μl of 10% sodium dodecyl sulfate was added and the contents in each well was dissolved in 10 ml of Aquasol (NEN) for scintillation counting. The results shown are representative of three experiments at different cell passsages. In all three panels the values are the mean of triplicate determinations, and the *bars* indicate standard deviations.

over the concentration range of 0.8–1.6 μM inhibited AL-CM and OSM stimulated migration of AIDS-KS cells. As discussed above regarding cell proliferation, the concentration of OSM in AL-CM is not high enough to account for the stimulation in cell growth and migration. The inhibitory effect on cell migration of ApoE-3 in the presence of AL-CM is likely due to competition with other chemotactic factors in AL-CM (28–30).

*Inhibition of Angiogenesis In Vivo.* The in vitro results led us to ask whether ApoE-3 had antiangiogenic properties in vivo. AIDS-KS cells were subcutaneously injected into athymic nude mice and ApoE-3 was administered intravenously. In these experiments (Fig. 3) ApoE-3 treatment produced a dose-dependent decrease in the size of the angiogenic KS-like lesions induced by subcutaneously injected AIDS-KS cells. The KS-like lesions in mice treated with 0.8 mg or 0.4 mg ApoE-3 were significantly smaller than the lesions of PBS-treated control mice, $p < 0.000008$ and $p = 0.0008$, respectively. Grossly, the lesions observed in mice treated with 0.8 or 0.4 mg of ApoE-3 (19) were pale in appearance compared to the erythematous lesions in the PBS and 0.2 mg ApoE-3–treated mice. The typical lesion observed histologically in the absence of ApoE-3 was characterized by spindle cells (primarily

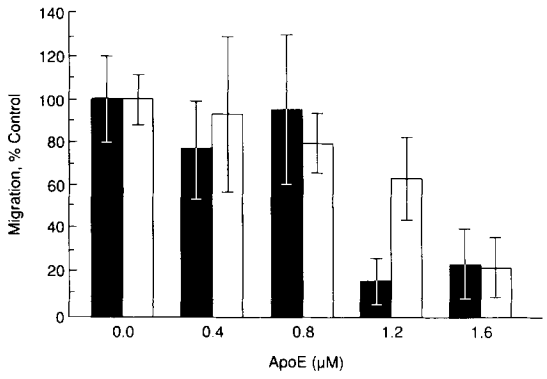


**Figure 2.** The effect of ApoE-3 on chemotaxis of AIDS-KS cells to OSM or AL-CM. Chemotaxis of the RW248 AIDS-KS cells was performed in a modified Boyden chamber assay. 8-μM pore polyvinlypyrrolidine-free nucleopore polycarbonate filters (Neuro Probe, Inc.) were coated with gelatin (0.1 mg/ml in 0.1% acetic acid). Subconfluent AIDS-KS cells were harvested on the first day after passage by brief treatment with 0.05% trypsin and 0.02% EDTA. The cells were reequilibrated for 2 h at room temperature in RPMI 1640 containing 10% FCS (Biofluids) and 20% AL-CM. Chemoattractants (OSM or AL-CM) and cells at $0.5 \times 10^6$ cells/ml were resuspended in RPMI 1640 containing 0.1% BSA (Sigma Chemical Co.). 28 μl of each chemoattractant was loaded into the lower chamber wells. 56 μl of cells ± ApoE-3 (at the indicated concentrations) were loaded in the upper chamber wells. Migration was performed at 37°C for 3.5 h. The filters were fixed, stained, and quantified microscopically by counting cells in three separate areas of each stained well. The data presented are from one representative experiment which was repeated at least three times with three different passages of cells. The number of cells migrating to OSM (■) at 25 ng/ml (140 ± 14) or 20% AL-CM (□) (168 ± 10) minus basal motility (59.5 ± 8) in the absence of inhibitor are defined as 100% of control. The nonstimulated migration determined at each ApoE-3 concentration was subtracted from stimulated motility and is expressed as percentage of the respective controls. The values are the mean of triplicate determinations and the *bars* indicate standard deviations.

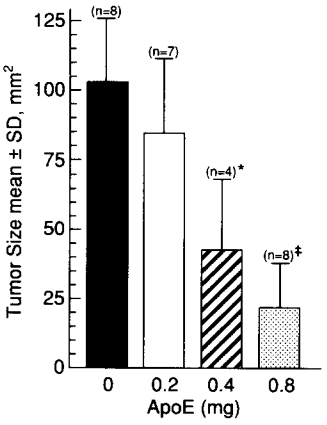


**Figure 3.** Intravenous ApoE-3 inhibits the formation of angiogenic lesions induced by AIDS-KS cells inoculated into athymic nude mice. 2 × $10^6$ AIDS-KS cells, RW248 passage 6-8, were transplanted subcutaneously on day 0 in the backs of male BALB/c *nu/nu* athymic mice. The mice were administered PBS (■), ApoE-3 0.2 mg (□), ApoE-3 0.4 mg (□), or ApoE-3 0.8 mg (□) on days 0–4 (*B*). On day 6 the animals were killed, and the tumor size was measured along with other macroscopic characteristics of the tumors. The values are representative of four in vivo experiments representing the mean and the error bars show the standard deviations. Statistical analysis was performed using the two-tailed Student's *t* test. *ApoE-treated mice at 0.4 mg vs. PBS-treated mice, $p = 0.0008$. ‡ApoE-treated mice at 0.8 mg vs. PBS-treated mice, $p < 0.000008$.

of murine origin), inflammatory cells, and neoangiogenesis (Fig. 4 *A*). In contrast, the ApoE-3–treated mice had either no lesions at the highest doses, or lesions devoid of spindle and inflammatory cells, and neoangiogenesis (Fig. 4 *B*).

In these experiments, ApoE-3 strongly inhibited proliferation and migration of the AIDS-KS cells, as well as the development of KS-like lesions in nude mice. These in vitro and in vivo results suggest a possible role for ApoE-3 in the regulation of AIDS-KS.

The most studied functions of ApoE include its role in cholesterol metabolism. ApoE is a polymorphic protein consisting of three isoforms, ApoE-2, E-3, and E-4 (18). These isoforms are the products of three alleles at a single gene locus. The normal allele, found in 60% of individuals, produces ApoE-3, whereas two mutant alleles result in the synthesis of ApoE-3 and E-4. These ApoE isoforms have varied effects on cholesterol metabolism. ApoE-3 is found in high density lipoproteins (HDL) and very low density lipoproteins (LDL) and mediates high affinity binding to the LDL receptor (18). Decreased levels of ApoE-3 are associated with an increased risk of atherosclerosis. ApoE-2 and E-4 bind the LDL receptor poorly, therefore individuals that express these isoforms have an increased risk of atherosclerosis. However, ApoE is synthesized by a number of cells that have no known role in cholesterol homeostasis, suggesting that ApoE may have other cellular functions depending upon the ApoE isoform expressed (18). For example, the expression of ApoE-4 is also associated with the development of late onset Alzheimer's disease (31, 32).

AIDS-KS (3–7) is a cytokine-mediated disease where inflammatory cytokines e.g., IL-1, IL-6, TNF-$\alpha$, OSM, and HIV-1 Tat proteins may cooperate in its induction and progression (28–30). Atherosclerosis (33, 34) is also a cytokine-mediated disease that is characterized by activated immune cells, smooth muscle hyperplasia, and altered cholesterol metabolism (33, 34). Activated lymphocytes and macrophages produce cytokines, including OSM, that stimulate the growth of intimal smooth muscle cells (35). OSM may therefore play a role in the pathogenesis of atherosclerosis (35), in addition to its role in the pathogenesis of AIDS-KS. We demonstrated in previous studies (20) that ApoE-3 inhibits smooth muscle proliferation in vitro. The ability of ApoE-3 to inhibit the proliferation and migration of AIDS-KS cells in vitro, as well as the inhibition of the development of KS-like lesions in nude mice suggest that ApoE-3 may function as a key regulator in AIDS-KS.

What are the ApoE levels in HIV-1–infected patients with KS as compared with other HIV-1–infected patients and age-matched controls? These ApoE levels have not been measured to date. However, the lipid abnormalities described in HIV-1–infected patients include increased triglycerides, decreased plasma LDL and HDL which constitutes an LDL B pheno-

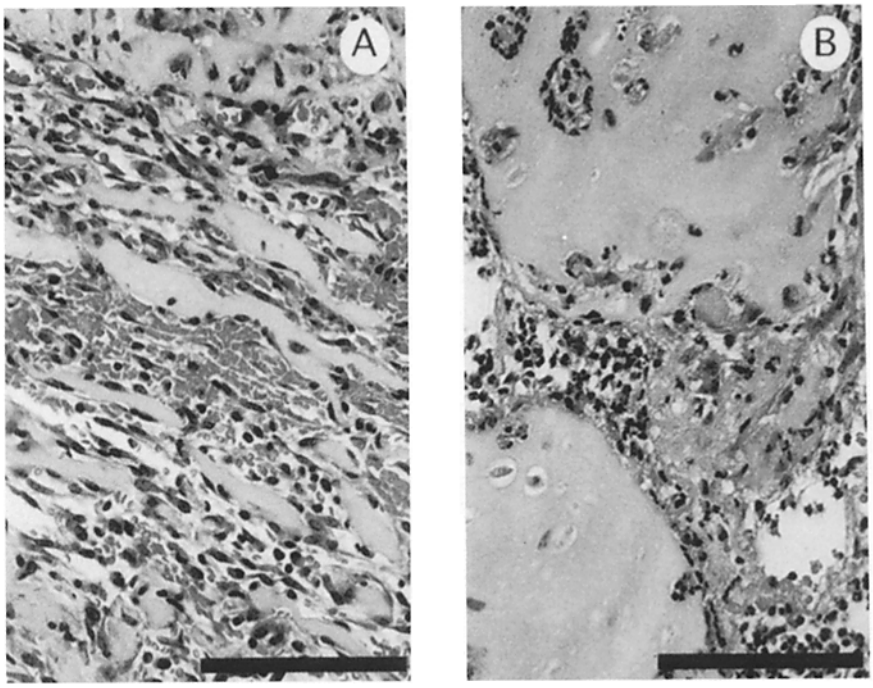


**Figure 4.** Histology of angiogenic lesions induced by KS cell strain RW248 in the absence of ApoE-3 (*A*) and presence of ApoE-3 (*B*). Male BALB/c *nu/nu* mice received (*A*) daily intravenous injections of normal saline and with (*B*) daily intravenous injections of 0.4 mg/mouse of ApoE-3. The in vivo experiments were performed as described in Fig. 3. On day 6 the angiogenic lesions were observed, measured, fixed in 10% formalin, paraffin embedded, cut, and stained with hematoxylin & eosin. The histology shown is from one representative experiment. *Bar*, 100 microns.

type (36). Persons with the LDL B phenotype have threefold increased risk of myocardial infarction when they acquire this atherogenic lipid profile (37). The LDL B phenotype is increased 2.5-fold in HIV-1–infected patients as compared with age matched controls (36) and is a result of HIV-1 infection causing an increase in serum IFN-$\alpha$ levels (38, 39). Plasma HDL levels decrease early in the clinical course of HIV-1–infected patients, before they develop clinical signs of AIDS (38). Since most of these studies arise from centers where the HIV-1–infected patients are primarily homosexual or bisexual males, we do not know if these lipid abnormalities are similar in all groups of HIV-1–infected patients. Our data suggests that a closer analysis of plasma ApoE protein isoform levels and other plasma lipid levels in patients with and without KS should be performed.

Effective treatments for AIDS-KS (3–7) include low dose combinations of doxorubicin, bleomycin, and vincristine or IFN-$\alpha$ (40–42). Most HIV-1–infected patients with KS are unable to tolerate aggressive chemotherapy due to hematologic and immunologic complications secondary to HIV-1 infection (40–42). As a result, it is very important that we develop effective therapies for AIDS-KS (3–7) that will not have the same spectrum of systemic toxicities as standard chemotherapeutic approaches (40–42).

In studies where 10 mg of nonrecombinant ApoE was administered intravenously three times per week to Watanabe rabbits over an 8.5-mo period there was no deterioration of kidney and liver functions or peripheral blood counts (43), although the size of the atherosclerotic plaques was significantly reduced. Pharmocokinetic studies (data not shown) of ApoE-3 in BALB/c *nu/nu* mice demonstrate that the half-life of ApoE-3 was 84 min. The liver and plasma had nearly identical peak concentrations of ApoE-3 due to rapid receptor-mediated uptake into the liver. ApoE-3 did not accumulate in other organs or fluids e.g., kidney or bile. This likely explains the lack of systemic toxicity of ApoE-3 in the Watanabe rabbits. In the in vivo KS model, where there is a blood supply developing from preexisting vessels, we would expect an accumulation of ApoE at this site. Based on the results presented above, we think that ApoE-3 (19) has potential as a novel therapeutic agent for the treatment of AIDS-KS.

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