

Anti-Kaposi's Sarcoma and Antiangiogenic Activities of Sulfated Dextrins

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Delivery of the sulfated polysaccharide dextrin 2-sulfate by the intraperitoneal route to the lymphatic circulation resulted in a clinically significant improvement in Kaposi's sarcoma in three patients. Our in vitro studies show that although sulfated dextrins do not interfere with the growth of isolated human umbilical vein endothelial cells, they do inhibit the morphological differentiation of endothelial cells into tubes as well as reduce new vessel formation in a placental angiogenesis assay. The antiangiogenic effect of dextrin 6-sulfate is greater than that of dextrin 2-sulfate and is independent of their anti-human immunodeficiency virus type 1 activities.

Kaposi's sarcoma is common in patients with AIDS (10). It is composed of thin-walled neovascular formations, inflammatory lymphocytes, and proliferating spindle cells (13, 23) whose growth is promoted by an abnormal cytokine and chemokine microenvironment. Current therapeutic approaches depend upon the distribution of the lesions and the degree of immunosuppression (28). Although some sulfated polysaccharides have been shown to have antiangiogenic activities in vitro (7, 9, 22, 33, 37), no clinical benefit has ever been demonstrated in patients with Kaposi's sarcoma.

Sulfated dextrins block the entry of human immunodeficiency virus type 1 (HIV-1) into lymphocytes and macrophages (11, 12, 30, 31). In a phase I and II clinical trial of one of these compounds with patients with late-stage AIDS, we demonstrated that the direct administration of dextrin 2-sulfate (D2S) into the lymphatic circulation by the intraperitoneal route resulted in a significant and sustained fall in the HIV-1 load (31). This was the first clinical trial to use the peritoneal route to target an antiretroviral agent directly to the lymphatic circulation. No clinical or biochemical toxicity was seen.

Three of the six patients treated with D2S had disseminated Kaposi's sarcoma. All showed clinical evidence of regression of their Kaposi's sarcoma lesions, even though they received no specific anti-Kaposi's sarcoma therapy. This improvement was gradual and was not like the rapid responses seen with chemotherapy. There was a reduction in the level of tumor-associated edema, the lesions developed a brown-tan halo, and the epithelium over the lesions desquamated (Fig. 1). In those areas where the Kaposi's sarcoma lesions were nodular and had ulcerated, the effect was healing of the ulcer, flattening of the nodular lesions, and formation of a hard, white, plaque-like surface. These changes were compatible with a response to therapy as defined by the AIDS Clinical Trials Group Oncology Committee (14). The occasional new lesion which arose was much smaller, paler, and flatter and grew very slowly.

During the clinical study, we collected the peritoneal dialysates from the patients after each 24-h period of treatment.

Cytospin preparations were either fixed with alcohol and stained with Papanicolaou stain or air dried, fixed with methanol, and stained with Giemsa stain. D2S accumulated in the peritoneal macrophages and mesothelial cells of treated patients but not in any other cell type found in the peritoneal cavity or in the peripheral blood of patients.

In subsequent in vitro experiments, many different cell types were cultured with 100 µg of endotoxin-free D2S per ml, 100 µg of biotinylated D2S per ml (31), 100 µg of dextrin 6-sulfate (D6S) per ml, 100 µg of dextrin per ml, or 100 µg of biotinylated dextrin per ml for up to 4 weeks (Table 1) in order to determine which cell types could internalize sulfated dextrins. The cells were then stained with 1,9-dimethylmethylene blue, which stains sulfated glycosaminoglycans (Blysan; Biocolor, Belfast, United Kingdom) or alkaline phosphatase-conjugated streptavidin and fast red-naphthol, which detect intracellular biotinylated sulfated dextrins. After 3 weeks of continuous culture with D2S and D6S, sulfated dextrins were found to accumulate only in peritoneal macrophages.

As sulfated dextrins did not accumulate in human umbilical vein endothelial cells (HUVECs) or in the Kaposi's sarcoma cell line KSY-1, we determined whether sulfated dextrins could interfere with the normal growth of these cells by an action at the level of the cell surface. Our previous studies of peripheral blood mononuclear cells and monocyte-derived macrophages have shown that D2S binds to the cell surface in a specific and saturable manner with a dissociation constant (K_d) of 82 ± 14 nM (30). Furthermore, concentrations up to 250 µg/ml did not affect cell count, cell viability, cell proliferation, or metabolic activity compared to those of cells cultured in the absence of each compound (11, 12).

Neither dextrin nor sulfated dextrins affected the rate of division of HUVECs or KSY-1 cells over a 7-day period, as determined by twice-weekly cell counts. Cell viability remained >95% at all times, as determined by trypan blue exclusion. The effects of sulfated dextrins on the growth of HUVECs and KSY-1 cells which were being cultured in the presence of basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) were also determined.

For these experiments, HUVECs (5×10^3 /well) were suspended in medium M199 containing 10% fetal calf serum, 2 mM L-glutamine, 100 IU of penicillin per ml, and 0.1 mg of streptomycin per ml. After 24 h, the medium was replaced with

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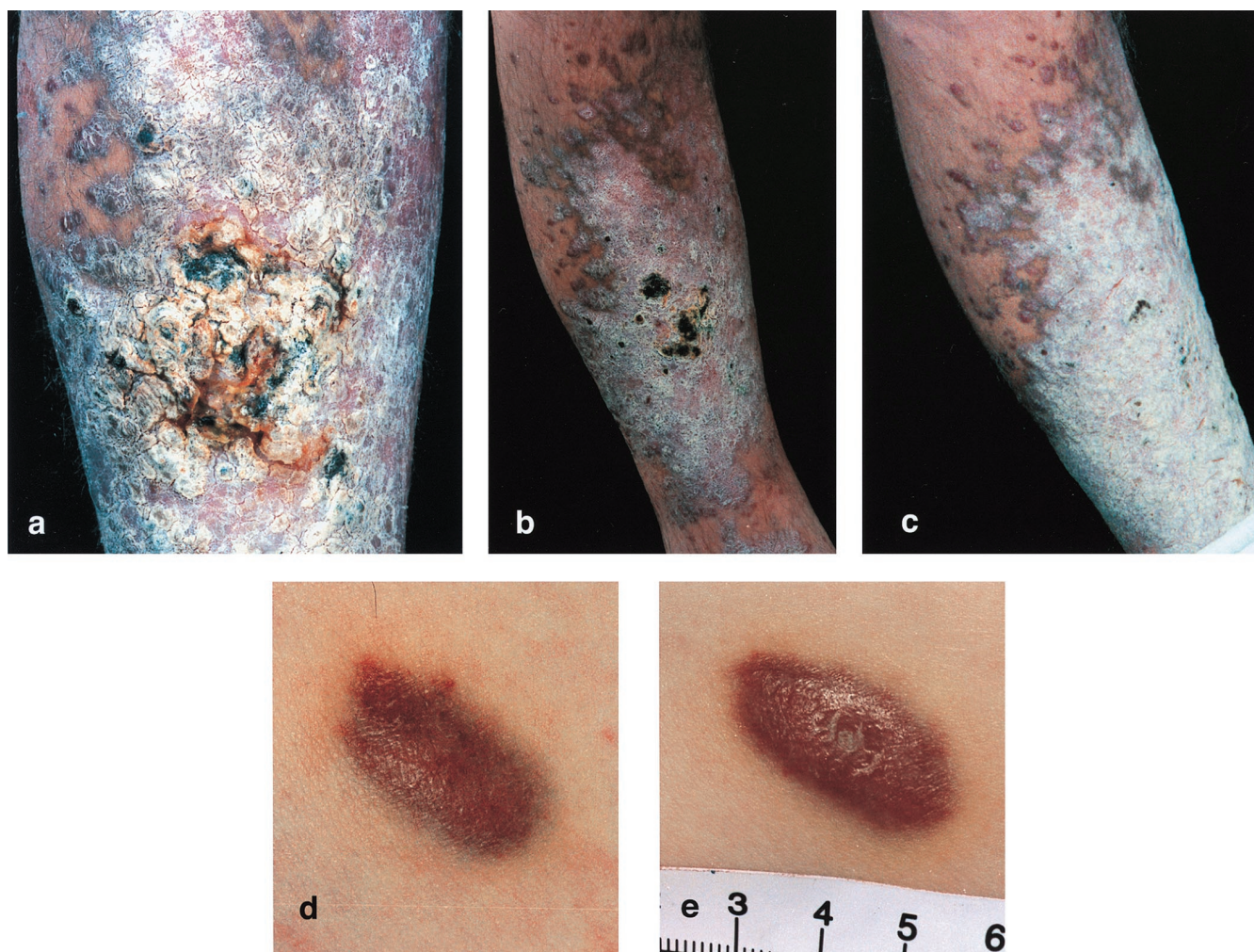


FIG. 1. Photographs of a patient were taken at 1 month (a), 3 months (b), and 4 months (c) after completion of a 28-day course of treatment with D2S. There was a reduction of tumor-associated edema, nodular lesions became flat, and the ulcerated areas healed with the formation of a hard, white, plaque-like surface. (d and e) Representative Kaposi's sarcoma lesions of two other patients 3 months after the completion of treatment. The lesions have darkened in color and have developed a brown-tan halo, and the epithelium over their surfaces has desquamated. No photographs were taken of the Kaposi's sarcoma lesions before the administration of D2S because we were not expecting to see a change in these lesions.

fresh medium containing either D2S (100 $\mu\text{g/ml}$) or D6S (100 $\mu\text{g/ml}$) for 1 h, following which either bFGF (10 ng/ml) or VEGF (20 ng/ml; Peprotech, Rocky Hill, N.J.) was added. These concentrations of VEGF and bFGF were chosen because they have been shown to cause HUVECs to proliferate (17, 36) and on the basis of our own dose titration studies. [^3H]thymidine (1 mCi/ml; Amersham Pharmacia, Little Chalfont, United Kingdom) was added 48 h later, and the cells were cultured for a further 16 h. Cells were harvested with an automated well harvester (Betaplate 96; Wallac Oy, Turku, Finland), and the radioactivity was counted with a Betaplate liquid scintillation counter. Sulfated dextrans did not alter the rate of proliferation of HUVECs or KSY-1 cells, as determined by cell counts and [^3H]thymidine incorporation, or affect the ability of exogenous bFGF or VEGF to cause proliferation (Fig. 2).

Given the anti-Kaposi's sarcoma activity observed in our patients and the lack of an effect of sulfated dextrans on endothelial cells or a Kaposi's sarcoma cell line in vitro, we investigated whether these molecules were interfering with the complex process of angiogenesis itself. The assay used was based upon the methods of Nicosia and Ottinetti (24) and Brown et

al. (3). Blood vessels (diameters, 1 to 2 mm) were excised from human placentas, cut into fragments, and cultured within a fibrin clot. ϵ -Amino-*n*-caproic acid was added twice weekly to prevent clot lysis. New vessel formation was quantified in a blind manner twice weekly by three different observers, with within-observer and between-observer variabilities of <10%. The presence of endothelial cells in new vessels was confirmed by using factor VIII (4) and CD31 antibodies (25).

Figures 3 and 4 show the effects of D2S and D6S on new vessel formation. No difference between vessels cultured in media and vessels cultured in the presence of dextrin (100 $\mu\text{g/ml}$) was seen (40 wells; two experiments; $P = 0.9$ on day 18). In contrast, there was a significant reduction in the number of new vessels which formed when D2S (100 $\mu\text{g/ml}$) was present. This was first seen at day 13 ($P = 0.02$), and the difference was still present at day 22 ($P = 0.03$). D6S also caused a significant decrease in new vessel formation. This was first seen at day 13 (60 wells; three experiments; $P = 0.004$) and was still present at day 22 ($P = 0.002$). The difference in the score obtained for D2S compared to that obtained for D6S was not statistically significant.

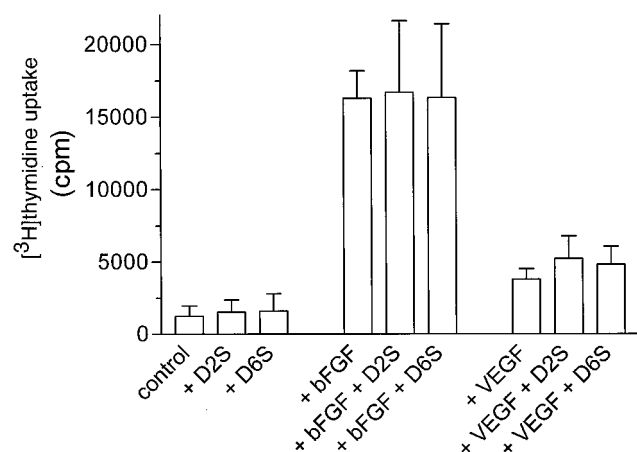


FIG. 2. The effects of sulfated dextrans on the proliferation of HUVECs induced by bFGF or VEGF were measured by a [^3H]thymidine incorporation assay. Neither D2S nor D6S had a significant effect on cellular proliferation, as determined by the [^3H]thymidine incorporation assay or as determined from cell counts (data not shown). In similar experiments with the KSY-1 cell line, bFGF and VEGF did not increase cellular proliferation and sulfated dextrans had no effect (data not shown).

As many cell types were present in the angiogenesis assay (e.g., endothelial cells, smooth muscle cells, and tissue macrophages), we then tried to establish whether sulfated dextrans had a specific effect on the ability of HUVECs to form into new vessels. Kubota et al. (15) have described an assay in which HUVECs can be induced to undergo rapid morphological differentiation into tubes which resemble capillary-like structures.

TABLE 1. Internalization of D2S and D6S by different cell types^a

Cell type (reference)	Sulfated dextrin internalized
Peritoneal macrophages.....	Yes
Peritoneal macrophages from patients with AIDS	Yes
Peripheral blood mononuclear cells.....	No
Monocytes.....	No
Monocyte-derived macrophages	No
HPB-ALL (21)	No
H9 (27).....	No
Jurkat (34)	No
U1 (8).....	No
U937 (32).....	No
U38 (2).....	No
HUVECs (35)	No
HeLa (19)	No
C11STH (5)	No
KSY-1 (18)	No
RBE 4 (6)	No
U87 (20).....	No

^a The cells were maintained in the presence of the sulfated dextrin for up to 4 weeks. Each cell type was tested on three separate occasions.

During this process, the cells remain viable and metabolically active and do not increase in number.

Human umbilical cord endothelial cells were isolated from umbilical cords as described previously (35) and were plated

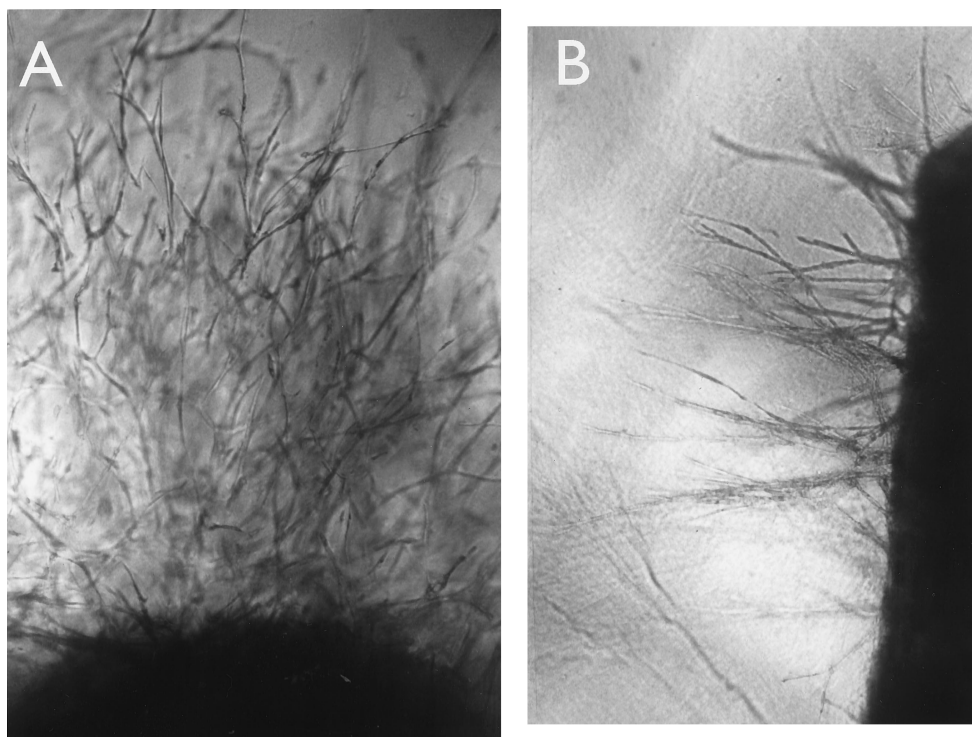


FIG. 3. New blood vessel formation at day 22. (a) Vessel cultured in medium 199 alone (score = 3); (b) vessel cultured in medium 199 and D2S (100 $\mu\text{g/ml}$) (score = 1). Magnification, $\times 40$.

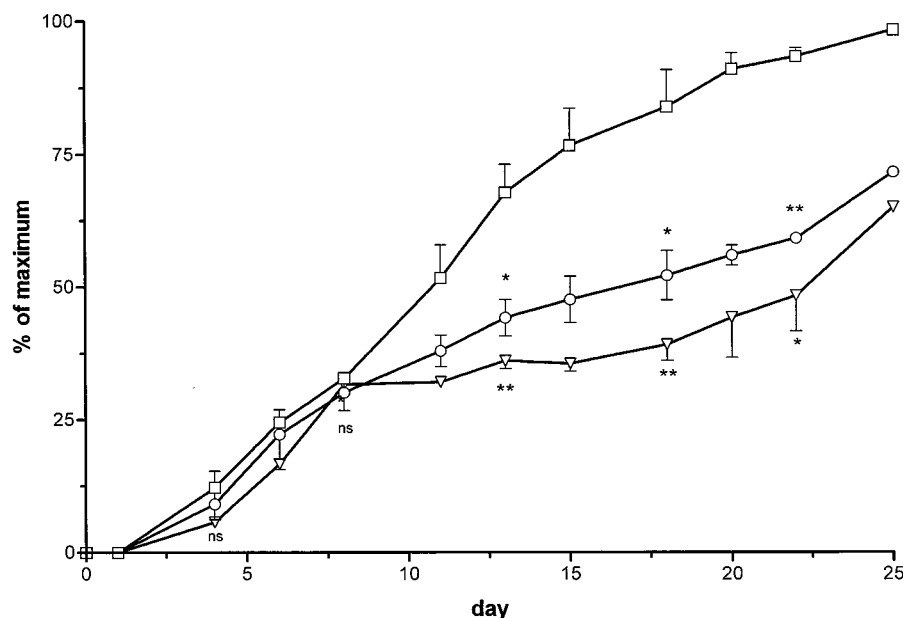


FIG. 4. D2S (○) and D6S (▽) reduced the rate of new vessel formation in an in vitro angiogenesis assay compared to that in medium alone (□). Compounds were tested at a final concentration of 100 $\mu\text{g/ml}$. The degree of new vessel formation was quantified in a blind manner twice a week by using a visual analog scale in which 0 equals no growth, 1 equals minimal new vessel formation, 2 equals significant new vessel formation, and 3 equals dense new vessel formation. An angiogenesis score was derived from each count by dividing the total score (i.e., the sum of scores for all the wells) by the maximum possible score and then expressing the result as a percentage. This graph is representative of three experiments with a total of 20 wells per plate per experiment for each compound tested. New vessel formation was quantified in a blind manner by three different observers twice a week, with within-observer and between-observer variabilities of $<10\%$. *P* values: ns (not significant), $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$.

(passages 3 to 6; 2×10^5 to 3×10^5 cells/ml) on Matrigel (Becton Dickinson)-coated tissue culture plates (1). Dextrin, D2S, or D6S was then added within a concentration range of 6.25 to 200 $\mu\text{g/ml}$. The result was read at 18 h (Fig. 5). D2S at 50 $\mu\text{g/ml}$ significantly reduced the level of tube formation, with

the effect being most marked at 200 $\mu\text{g/ml}$ (Fig. 5 and Table 2; $n = 3$). Tube formation was significantly reduced with D6S at 12.5 $\mu\text{g/ml}$ and was abolished with D6S at 100 $\mu\text{g/ml}$. Cell viability remained $>95\%$ at all times.

Our original observations of an anti-Kaposi's sarcoma effect

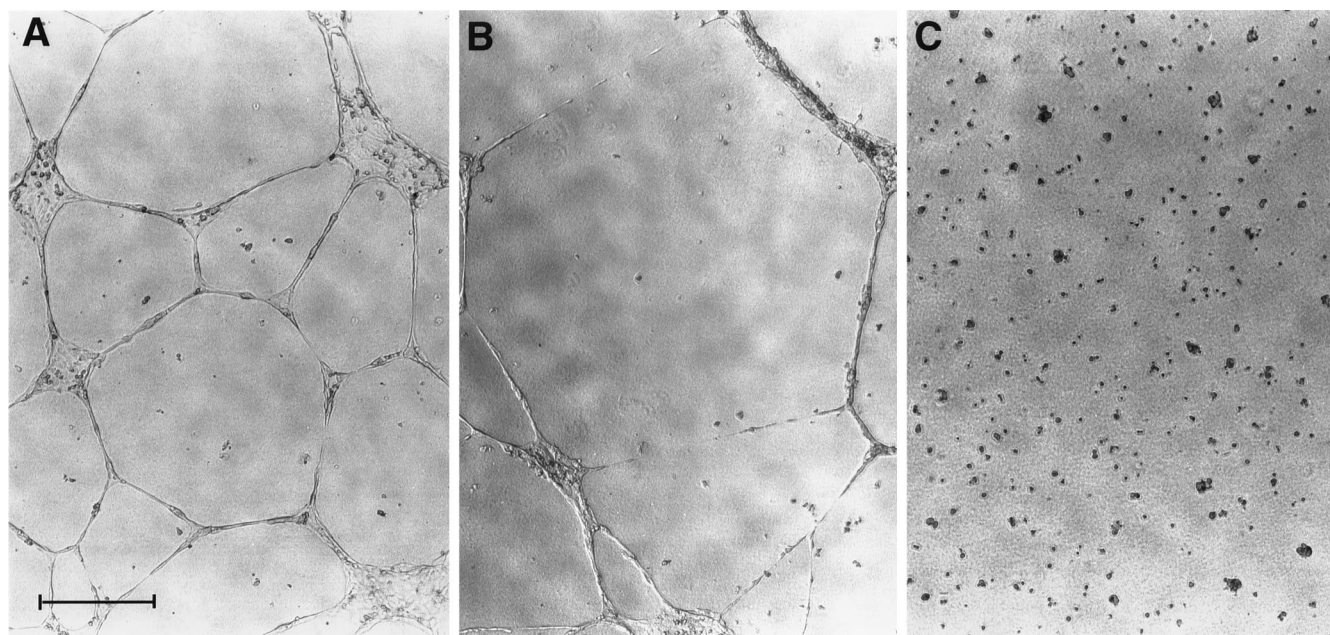


FIG. 5. Endothelial microtubule formation in the presence of sulfated dextrans. A visual analogue scale was used to determine the extent of tube formation, which was scored on a scale from 0 (all cells remain single) to 4 (all cells are involved in tubular structures) as described previously (1, 17). Bar, 100 μm . (A) Control well in the presence of dextrin (100 $\mu\text{g/ml}$); (B) D2S present at 200 $\mu\text{g/ml}$; (C) D6S present at 100 $\mu\text{g/ml}$. Magnifications, $\times 33$.

TABLE 2. Endothelial microtubule formation in the presence of sulfated dextrans^a

Compound (concn [μ g/ml])	Tube formation score
Dextrin	4
D2S	
0	4
6.25	4
12.5	4
25	4
50	3
100	3
200	2
D6S	
0	4
6.25	4
12.5	3
25	2
50	1
100	0
200	0

^a A visual analogue scale was used to determine the extent of tube formation, which was scored on a scale from 0 (all cells remain single) to 4 (all cells are involved in tubular structures) as described previously (1, 15). New vessel formation was quantified in a blind manner by three different observers at 18 h. The within-observer and between-observer variabilities were <10%. There was no significant increase in the number of HUVECs during the course of the experiment, and cell viability was always >95%, as determined by trypan blue exclusion ($n = 3$).

of D2S were made for three patients. Although recent reports have suggested that a reduction in the HIV-1 load can lead to the regression of Kaposi's sarcoma (16), this is unlikely to explain our clinical observations with sulfated dextrans for two reasons. First, the viral load in our patients did not fall to the very low levels required (i.e., a plasma HIV-1 RNA level of <400 copies/ml) and, second, because there was no recovery of the CD4 cell count in our patients (31).

Sulfated dextrans were internalized by peritoneal macrophages both in vitro and in vivo but not by endothelial or epithelial cells. An intracellular mechanism of action for their antiangiogenic activities therefore seems most unlikely. D2S bound to the surface of HeLa cells in a specific and saturable manner with a K_d of 107 ± 12 nM and a maximum binding (B_{max}) of 4.1 ± 0.2 pmol/ 10^6 cells (30). Although this did not alter the rate of proliferation of HeLa cells or of HUVECs, even in the presence of bFGF or VEGF, it did interfere with the ability of HUVECs to undergo morphological differentiation into tubes that resembled capillaries when they were plated on Matrigel. It also reduced the level of new vessel formation in a placental angiogenesis assay. These observations suggest that the antiangiogenic effects of sulfated dextrans are mediated at the level of the cell surface, possibly by an effect on endothelial cell migration.

The biological assays by which sulfated dextrans were tested fulfilled the two hallmarks of angiogenesis, i.e., endothelial cell proliferation and capillary sprouting (29). Although D2S and D6S both reduce the levels of tube formation and capillary sprouting, the effect on tube formation was most marked with D6S. In this respect, pentosan polysulfate is notable because it has also undergone detailed study. Daily intraperitoneal injections were well tolerated by athymic nude mice and prevented the growth of a subcutaneous adrenal tumor, provided that treatment was started before tumor inoculation. A delay in the

administration of pentosan polysulfate reduced the incidence of new tumors by only 50% and had no effect on tumors which were already well established. However, when patients with AIDS and Kaposi's sarcoma were treated with intravenous pentosan polysulfate, they showed no objective clinical response (26). The investigators suspected that the molecular weight and strong anionic nature of pentosan polysulfate resulted in its confinement to the systemic vascular circulation.

We have previously shown that pentosan polysulfate has much less anti-HIV-1 activity than D2S and that it does not bind to the same site on the cell surface as D2S (30). Our studies also suggest that sulfated dextrans do not interfere with the proliferative activity of either bFGF, as has been shown for pentosan polysulfate (17), or VEGF. Therefore, although sulfated dextrans and pentosan polysulfate have anti-HIV-1 activities and they inhibit angiogenesis, their mechanisms of action appear to be different. Sulfated dextrans could be interfering with the paracrine mechanisms by which molecules such as chemokines trigger new vessel formation in the lymphatic circulation.

Our in vitro and in vivo observations show that sulfated dextrans inhibit tube formation and new vessel formation and that they slow the clinical progression of Kaposi's sarcoma. It should now be possible to develop more powerful inhibitors of angiogenesis which can be safely and effectively delivered to the lymphatic circulation.

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