Chemically sulfated *Escherichia coli* K5 polysaccharide derivatives as extracellular HIV-1 Tat protein antagonists

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Received 12 February 2004; revised 13 May 2004; accepted 19 May 2004

Available online 31 May 2004

Edited by Hans-Dieter Klenk

Abstract The HIV-1 transactivating factor (Tat) acts as an extracellular cytokine on target cells, including endothelium. Here, we report about the Tat-antagonist capacity of chemically sulfated derivatives of the *Escherichia coli* K5 polysaccharide. *O*-sulfated K5 with high sulfation degree (K5-OS(H)) and *N*,*O*-sulfated K5 with high (K5-N,OS(H)) or low (K5-N,OS(L)) sulfation degree, but not unmodified K5, *N*-sulfated K5, and *O*-sulfated K5 with low sulfation degree, bind to Tat preventing its interaction with cell surface heparan sulfate proteoglycans, cell internalization, and consequent HIV-LTR-transactivation. Also, K5-OS(H) and K5-N,OS(H) prevent the interaction of Tat to the vascular endothelial growth factor receptor-2 on endothelial cell (EC) surface. Finally, K5-OS(H) inhibits $\alpha_v\beta_3$ integrin/Tat interaction and EC adhesion to immobilized Tat. Consequently, K5-OS(H) and K5-N,OS(H) inhibit the angiogenic activity of Tat in vivo. In conclusion, K5 derivatives with distinct sulfation patterns bind extracellular Tat and modulate its interaction with cell surface receptors and affect its biological activities. These findings provide the basis for the design of novel extracellular Tat antagonists with possible implications in anti-AIDS therapies.

Keywords: HIV; Tat; K5 polysaccharide; Antagonist; Polyanion

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Abbreviations: bTat, biotinilated Tat; CAT, chloramphenicol acetyltransferase; CAM, chick embryo chorioallantoic membrane; CHO cells, Chinese hamster ovary cells; ECs, endothelial cells; FGF, fibroblast growth factor; GAG, glycosaminoglycan; GFP, green fluorescent protein; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, *N*-acetyl-glucosamine; GST, glutathione *S*-transferase; HSPGs, heparan sulfate proteoglycans; KDR, vascular endothelial growth factor receptor 2; K5-NS, *N*-sulfated K5; K5-OS(L), *O*-sulfated K5 with low degree of sulfation; K5-OS(H), *O*-sulfated K5 with high degree of sulfation; K5-N,OS(L), *N*,*O*-sulfated K5 with low degree of sulfation; K5-N,OS(H), *N*,*O*-sulfated K5 with high degree of sulfation; LTR, long terminal repeats; xcTat, extracellular Tat; VEGF, vascular endothelial growth factor; VN, vitronectin

1. Introduction

The transactivating factor of the HIV-1 virus (Tat) is released from HIV-1 infected cells [1]. Extracellular Tat (xcTat) elicits different biological activities in several cells types including endothelium, thus contributing to the onset of AIDS-associated pathologies [2,3].

The mechanisms by which xcTat stimulates target cells are manifold. Tat binds cell-surface heparan sulfate proteoglycans (HSPGs), being then internalized in a bioactive form to reach the nucleus and transactivate cellular and viral genes [4]. Commercial heparin binds xcTat and inhibits its interaction with cell-associated HSPGs, its cellular uptake and transactivation [4–6]. Tat/heparin interaction requires a proper tridimensional conformation of the protein and at least some 2-*O*-, 6-*O*-, and *N*-positions of the glycosaminoglycan (GAG) to be sulfated [7]. Sulfated groups of heparin bind to a stretch of positively charged amino acid residues present in the basic domain of Tat [5]. Other polyanionic compounds such as suramin and its derivatives, dextrin-2-sulfate, and pentosan polysulfate bind Tat and inhibit its interaction with cell surface HSPGs and consequent biological activities [3] (and reference therein).

xcTat also binds various signaling receptors in target cells, including the vascular endothelial growth factor receptor (Flk-1/KDR) [8] and integrins [2,9]. In endothelial cells (ECs), the interaction of xcTat with KDR and/or $\alpha_v\beta_3$ integrin mediates Tat-induced cell adhesion, proliferation and chemotaxis in vitro and neovascularization and vascular permeability in vivo [2]. Despite the importance of these observations about the multiple mechanisms of action of xcTat, only a few data, if any, are available about the possible effects of heparin-like Tat antagonists on these interactions.

The capsular K5 polysaccharide from *Escherichia coli* has the same structure $[\rightarrow 4)$-β-D-GlcA-$(1\rightarrow 4)$-α-D-GlcNAc-$1(1\rightarrow]_n$ as the heparin precursor *N*-acetyl heparosan [10] in which GlcA is glucuronic acid and GlcNAc is *N*-acetyl-glucosamine. Previous studies demonstrated the possibility to generate sulfated K5 derivatives by chemical sulfation in *N*- and/or *O*-positions [11]. Interestingly, K5 derivatives with defined sulfation patterns interact with the angiogenic fibroblast growth factor-2 (FGF-2) and FGF-8, affecting their receptor

0014-5793/$22.00
doi:10.1016/j.febslet.2004.05.033

interaction, signal transduction and biological activity [12,13]. Also, sulfated K5 derivatives inhibit the replication of HIV in T cells and macrophages by preventing virion attachment and entry [14].

On these bases, a panel of *N*-, *O*-, and *N*,*O*-sulfated K5 derivatives with different sulfation patterns were evaluated for their ability to bind Tat, to affect its interaction with cell surface receptors and its biological activity in vitro and in vivo. The results demonstrate that selective sulfation patterns confer different Tat antagonist capabilities to K5 derivatives.

2. Materials and methods

2.1. Reagents

86 amino acid HIV-1 Tat was expressed and purified by *E. coli* as glutathione *S*-transferase fusion protein (GST–Tat) or as GST–Tat fused to the green fluorescent protein (GST–Tat–GFP) [6]. GST and GFP moieties do not interfere with the heparin-binding capacity, long terminal repeat (LTR)-transactivating activity and angiogenic activity of Tat [15]. Synthetic Tat and its biotinilated form (bTat) were from Tecnogen (Caserta, Italy). The anti-$\alpha_v\beta_3$ monoclonal LM 609 antibody was from Chemicon International (Temecula, CA). The anti-KDR antibody was from Prof. H.A. Weich, Max Plank Institute, Germany. Human recombinant vascular endothelial growth factor $(VEGF)_{165}$ was from Calbiochem Biochemicals (La Jolla, CA). The homogeneously sized 24-mer heparin was provided by U. Lindahl (Uppsala University, Sweden). K5 polysaccharide derivatives were obtained by *N*-deacetylation/*N*-sulfation and/or *O*-sulfation of a single batch of K5 polysaccharide [12].

2.2. BIAcore binding assays

A BIAcore X apparatus (BIAcore Inc, Piscataway, NJ) was used. Surface plasmon resonance (SPR) was exploited to measure changes in refractive index caused by the ability of the different GAGs to bind to GST–Tat immobilized to a BIAcore sensorchip. To this purpose, 50 μg/ml of GST–Tat were allowed to react with a flow cell of a CM5 sensorchip that was previously activated with 50 μl of a mixture of 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride and 0.05 M *N*-hydroxysuccinimide. These experimental conditions allowed the immobilization of 10 281 resonance units (RUs), corresponding to approximately 0.3 pmol of GST–Tat. Similar results were obtained for the immobilization of GST, here used as a negative control and for blank subtraction. Increasing concentrations of the various K5 derivatives in 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4 (HBS) were then injected over the GST–Tat or GST surfaces for 4 min. (to allow their association with immobilized proteins) and then washed until dissociation was observed. After every run, the sensorchip was regenerated by injection of HBS containing 2.0 M NaCl. No difference in the binding parameters was found when sensorgrams obtained from the injection of K5 derivatives on GST surface were used as a blank to correct for possible aspecific binding (data not shown). In parallel experiments, SPR was exploited to evaluate the capacity of *O*-sulfated K5 with high degree of sulfation (K5-OS(H)) to compete with sensorchip-immobilized heparin for the binding to free GST–Tat protein. To this purpose, heparin was biotinilated on its reducing end and a flow cell of an F1 sensorchip was activated with streptavidin. Then, biotinilated heparin was allowed to react with the streptavidin-coated sensorchip as already described [15]. GST–Tat alone or in the presence of increasing concentrations of K5-OS(H) was then injected over the heparin surface for 5 min (to allow the association of the protein with heparin) and then washed until dissociation was observed. The SPR signal was expressed in terms of RUs.

2.3. Preparation of ^{3}H-labeled heparin and GST–Tat affinity chromatography

^{3}H-heparin was prepared as described [16] and loaded (50 μg/300 μl) onto a 80 μl GST–Tat–glutathione–agarose column in the presence of the various GAGs. The column was then washed with PBS and eluted with 2.0 M NaCl. Radioactivity in the eluate was measured in a liquid scintillation counter.

2.4. Cell cultures

Wild type and HSPG-defective A745 CHO-K1 cells [17] (provided by J.D. Esko, University of Birmingham, AL) were grown in Ham's F 12 medium (Gibco, Grand Island, NY) with 10% FCS (Gibco). HL3T1 cells, derived from HeLa cells, contain integrated copies of pL3CAT plasmid in which the *chloramphenicol acetyltransferase* (CAT) bacterial gene is driven by HIV-1 LTR [18]. They were grown in Dulbecco's modified Eagle's medium (Gibco) with 10% FCS. Porcine aortic ECs (PAECs) and KDR-transfected PAECs (PAEC/KDRs) [19] were grown in Ham's F-12 medium with 10% FCS and 2% glutamine. Transformed fetal bovine aortic endothelial GM 7373 cells [20] were grown in Eagle's minimal essential medium (Gibco) with 10% FCS, vitamins, essential and non-essential amino acids. Human umbilical vein ECs (HUVECs) were from Biowhittaker (Walkersville, MA) and cultured in EGM-2 medium (Biowhittaker).

2.5. Cell-binding assay of bTat

Subconfluent cultures of the different cell lines in 96-wells plates were incubated for 2 h at 4 °C in PBS containing 0.1 mg/ml $CaCl_2$, 0.1 mg/ml $MgCl_2$, 0.1% gelatin and bTat (600 ng/ml) in the presence of the different GAGs. At the end of incubation, Chinese hamster ovary (CHO) cells and PAECs were washed with PBS alone or containing 2.0 M NaCl, respectively. The amount of cell-associated bTat was detected with horseradish peroxidase-labeled avidin (1/1500) and chromogen substrate ABTS (Kirkgaard & Perry Laboratories, Gaithersburg, MD).

2.6. HIV-LTR transactivation and Tat internalization assays

The two assays were performed on HL3T1 cells exactly as described [6].

2.7. Cell-free $\alpha_v\beta_3$ integrin/GST–Tat interaction and cell adhesion assay

Human $\alpha_v\beta_3$ integrin purification, cell-free $\alpha_v\beta_3$ integrin/GST–Tat interaction and cell adhesion assay with GM 7373 ECs were performed as previously described [21].

2.8. Chick embryo chorioallantoic membrane assay

Chick embryo chorioallantoic membrane (CAM) assay was performed as described [22]. Briefly, a window was opened in the egg shell of three-day-old fertilized chicken eggs. At day 8, gelatin sponges containing Tat in the absence or in the presence of K5 derivatives were implanted on the CAMs (10 embryos per group). At day 12, the angiogenic response was scored by counting the number of vessel that surround the sponge.

3. Results

3.1. Interaction of sulfated K5 derivatives with Tat protein

Unmodified K5 and sulfated K5 derivatives were evaluated for their ability to bind Tat by using the BIAcore technology. As shown in Fig. 1A, when injected at 30 nM, K5-OS(H) and *N*,*O*-sulfated K5 with high degree of sulfation (K5-N,OS(H)) bind to GST–Tat immobilized onto a BIAcore sensorchip with high capacity (200–250 RU bound at the end of the injection phase). Under the same experimental conditions, *N*,*O*-sulfated K5 with low degree of sulfation (K5-N,OS(L)) and heparin (data not shown) were less effective (60–70 RU bound), whereas unmodified K5, *N*-sulfated K5 (K5-NS), and *O*-sulfated K5 with low degree of sulfation (K5-OS(L)) did not show a significant binding capacity also when tested at 300 μM (data not shown). Specificity of the interaction was demonstrated by the lack of binding of K5-N,OS(H) and K5-OS(H) to a GST–coated sensorchip (Fig. 1B).

Increasing concentrations of K5-N,OS(H) (Fig. 1C), K5-N,OS(L), K5-OS(L), K5-OS(H) or heparin (not shown) where injected over the GST–Tat surface to evaluate the binding parameters (Table 1). In some experiments, the association phase of GAG/GST–Tat interaction was allowed to proceed to

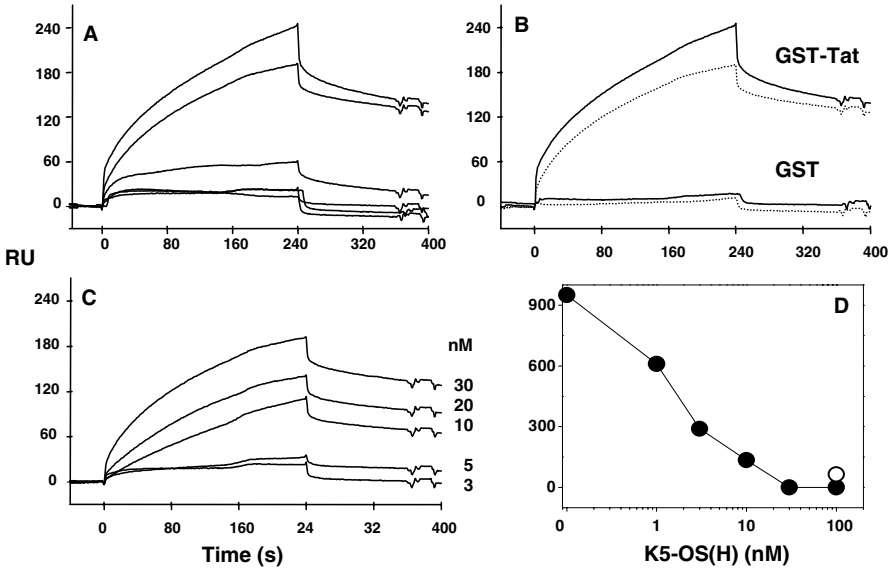

Fig. 1. Interaction of K5 derivatives with Tat: (A) K5 derivatives were injected at 30 nM over a BIAcore sensorchip containing GST–Tat. From top to bottom: K5-OS(H), K5-N,OS(H), K5-N,O-S(L), K5-OS(L), K5 and K5-NS. (B) K5-OS(H) (straight line) or K5-N,OS(H) (dotted line) (both at 30 nM) were injected over GST–Tat or GST surfaces. (C) Increasing concentrations of K5-NOS(H) were injected over a GST–Tat surface. (D) GST–Tat (125 nM) was injected over a flow cell of a BIAcore sensor chip containing streptavidin plus immobilized biotinilated heparin in the presence of increasing concentrations of K5-OS(H) (●). In parallel experiments, GST–Tat (125 nM) was injected alone and allowed to interact with the heparin surface for 5 min. Then, K5-OS(H) (100 nM) was injected (○). The response (in RU) in the different experimental conditions was recorded at the end of injections and plotted as a function of K5-OS(H) concentration.

Table 1
Binding parameters of the interaction of K5 derivatives to GST–Tat immobilized to a BIAcore sensorchip

Sample	Molecular weight (kDa)	Association rate (k_{ass}) (1/Ms)	Dissociation rate (k_{diss}) (1/s)	Dissociation constant (K_d) (nM)	
				Kinetics	Equilibrium
K5	30.0	n.d.	n.d.	n.d.	n.d.
K5-NS	15.0	n.d.	n.d.	n.d.	n.d.
K5-OS(L)	14.0	n.d.	n.d.	n.d.	n.d.
K5-OS(H)	11.0	$2.53 \pm 1.2 \times 10^{6}$	$3.97 \pm 2.2 \times 10^{-3}$	2.0	7.05
K5-N,OS(L)	13.0	$3.4 \pm 0.5 \times 10^{5}$	$6.28 \pm 2.5 \times 10^{-3}$	18.0	69.9
K5-N,OS(H)	15.0	$4.9 \pm 2.1 \times 10^{5}$	$6.16 \pm 3.0 \times 10^{-4}$	10.0	16.7
Heparin	13.6	$1.4 \pm 1.1 \times 10^{5}$	$2.83 \pm 0.8 \times 10^{-3}$	20.0	16.0

Binding parameters were calculated by the non-linear curve fitting software package BIAevaluation 3.2 using a single site model with correction for mass transfer. Only sensorgrams whose fitting gave values of X^2 close to 10 were used [29]. Association and dissociation rates were calculated separately using low (1–5 nM) and high (30–300 nM) concentrations of K5 derivatives, respectively [30]. Each data is the mean ± S.E.M. of 3–4 independent determinations. Dissociation constant (K_d) was instead derived from the k_{diss}/k_{ass} ratio (kinetics). Alternatively, data from the equilibrium binding between GAGs and GST–Tat were used to calculate an affinity value independent of the kinetics of binding (equilibrium).
The correlation coefficient of the linear regression of the equilibrium binding data was always higher than 0.8 (n.d.: non detectable).

equilibrium and the data were used to calculate an affinity value independent of the kinetics of binding. This analysis demonstrates that GST–Tat/heparin interaction occurs with a K_d equal to 16 nM, a value consistent with our kinetics analysis (20 nM) and with previous determinations obtained with different experimental approaches [6,15]. Interestingly, K5-OS(H) binds immobilized GST–Tat with an affinity that is 5–10 times higher than that of the other GAGs tested (Table 1).

K5 derivatives are expected to act as free antagonist that interfere with the binding of exTat to cell-surface associated HSPGs. On this basis, we evaluated the capacity of free K5-OS(H) to sequester Tat in the mobile phase thus inhibiting its interaction with heparin immobilized onto a BIAcore sensorchip, an experimental condition that resembles Tat interaction with cell-surface HSPGs [15]. As shown in Fig. 1D, when injected together with Tat on the heparin surface, K5-OS(H) prevents Tat/heparin interaction in a dose-dependent manner. In parallel experiments, Tat was allowed to reach a binding equilibrium with the heparin surface. Then, K5-OS(H) was injected on the sensorchip. Under these experimental conditions, K5-OS(H) disrupts the already established Tat-heparin complex (Fig. 1D).

Next, sulfated K5 derivatives were assessed for their capacity to compete with ^{3}H-heparin for the binding to Tat. K5-OS(H), K5-N,OS(L), K5-N,OS(H) and unlabeled heparin competed with ^{3}H-heparin for the binding to immobilized GST–Tat. In contrast, unmodified K5, K5-NS, and K5-OS(L) did not exert a significant competition when tested at 10 μg/ml (Fig. 2).

3.2. Effect of K5 derivatives on Tat/HSPG interaction

The capacity of sulfated K5 derivatives to affect xcTat/HSPG interaction in CHO-K1 cells was then evaluated. A745

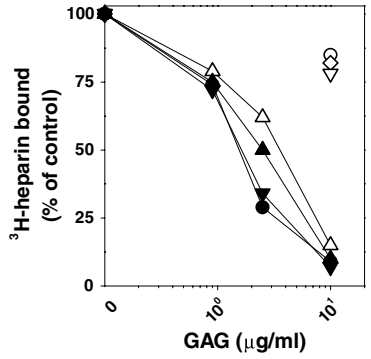

Fig. 2. Competition binding assays: GST–Tat–glutathione–agarose columns were loaded with ^{3}H-heparin in the presence of K5 (○), K5-NS (◇), K5-OS(L) (▽), K5-OS(H) (▼), K5-N,OS(L) (△), K5-N,OS(H) (▲) or heparin (●). Columns were washed and bound radioactivity measured. Each point is the mean of 3 determinations in duplicate. S.E.M. never exceeded 12% of the mean value.

CHO-K1 cell mutants defective for HSPG synthesis were used as a control for non-specific Tat interaction [4]. bTat binds CHO-K1 cells and a 2.0 M NaCl wash reduces the amount of cell surface-bound Tat to values similar to those measured in HSPG-deficient A745 CHO-K1 mutants (Fig. 3A). This confirms that the observed binding is mainly due to bTat/HSPGs interaction [23,24]. Heparin, K5-OS(H), K5-N,OS(L), and K5-N,OS(H) were all able to inhibit Tat/HSPG interaction in CHO-K1 cells. Unmodified K5, K5-NS, and K5-OS(L) were instead ineffective (Fig. 3B).

Tat/HSPG interaction leads to Tat internalization and gene transactivation [4]. In agreement with surface binding experiments performed in CHO-K1 cells, heparin, K5-OS(H), K5-N,OS(L), and K5-N,OS(H) inhibited the internalization of GST–Tat–GFP in HL3T1 cells (Fig. 3C). Accordingly, K5-OS(H), K5-N,OS(L), and K5-N,OS(H) inhibited the LTR-transactivating activity of GST–Tat in a dose-dependent manner with a potency similar to that shown by heparin (Fig. 3D). Unmodified K5, K5-NS, and K5-OS(L) were ineffective in both the assays (Fig. 3C and D).

Finally, K5-OS(H) also inhibits LTR transactivation exerted by exogenously added synthetic Tat in HL3T1 cells, ruling out a possible aspecific action of K5 derivatives on the GST or GFP moieties of the Tat fusion proteins used in the previous assays (Fig. 3D). On the contrary, K5-OS(H) did not affect HIV-LTR transactivation exerted by native Tat produced endogenously by HL3T1 cells following transient transfection with an expression vector harboring the HIV-1 Tat cDNA (data not shown), supporting the hypothesis of an extracellular mechanism of action of K5 derivatives.

3.3. Effect of K5 derivatives on Tat/KDR interaction

Binding and activation of KDR is required for Tat-induced EC proliferation and chemotaxis in vitro and for neovascularization and vascular permeability in vivo [8,25]. bTat binds the surface of KDR-overespressing PAEC/KDR cells with high capacity when compared to parental cells (Fig. 4A). The binding resists a 2.0 M NaCl wash but is prevented by suramin, anti-KDR antibodies, and a molar excess of $VEGF_{165}$, in keeping with an high affinity binding to KDR receptor [8]. K5

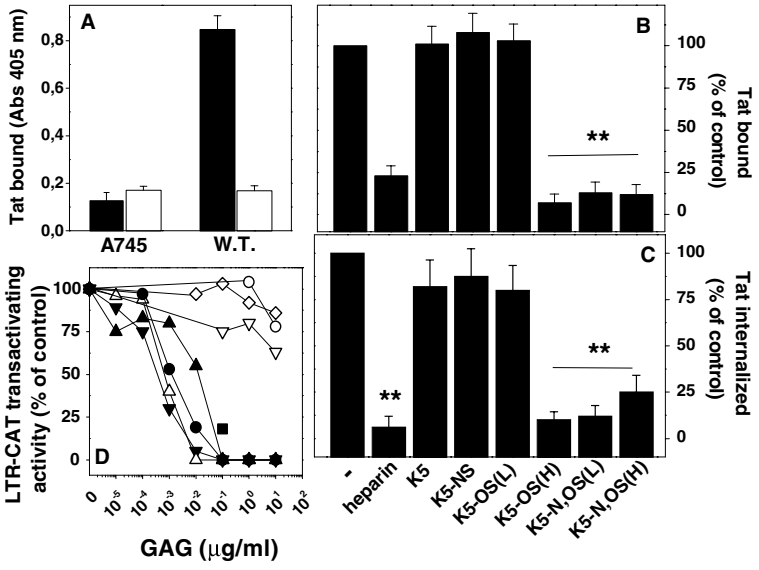

Fig. 3. Effect of K5 derivatives on Tat binding to cell surface HSPGs, cell internalization and HIV-LTR transactivation: (A) A745 CHO-K1 cells (A745) and wild type CHO-K1 cells (W.T.) were incubated with bTat and then washed with PBS alone (black bars) or PBS plus 2 M NaCl (white bars). (B) Wild type CHO-K1 cells were incubated with bTat in the absence (−) or in the presence of the indicated GAGs (100 ng/ml). Then, the amount of cell-associated bTat was measured. In panel B, data are expressed as percent of bTat bound to control cells in the absence of GAGs. Each bar represents the mean ± S.E.M. of three determinations in duplicate. Student's *t*-test: *, $P < 0.001$. (C) HL3T1 cells were treated with GST–Tat-GFP (400 ng/ml) in the absence (−) or in the presence of the indicated GAGs (10 ng/ml). Then, the amount of internalized GST–Tat-GFP was evaluated. Data are expressed as percent of GST–Tat-GFP internalized in the absence of GAGs. Each bar represents the mean ± S.E.M. of 3–4 determinations in duplicate. Student's *t*-test: *, $P < 0.001$. (D) HL3T1 cells were incubated with GST–Tat (200 ng/ml) in the presence of K5 (○), K5-NS (◇), K5-OS(L) (▽), K5-OS(H) (▼), K5-N,OS(L) (△), K5-N,OS(H) (▲), or heparin (●). Alternatively, cells were treated with synthetic Tat (■) devoid of the GST or GFP moiety in the absence or in the presence of K5-OS(H) (100 ng/ml). Then, cell extracts were assayed for the levels of CAT antigen. Data are expressed as percent of the activity measured in the absence of GAGs. Each point is the mean of 4–5 determinations in duplicate. S.E.M. never exceeded 13% of the mean value. Student's *t*-test: **, $P < 0.01$.

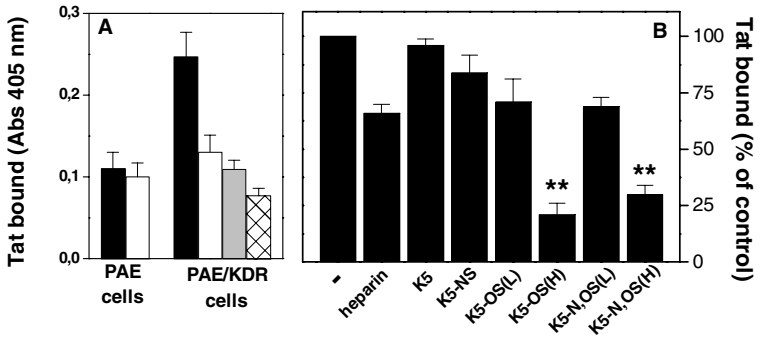

Fig. 4. Effect of K5 derivatives on Tat/KDR interaction. (A) Parental PAE cells and PAEC/KDR cells were treated with bTat in the absence (black bars) or in the presence of suramin (300 μg/ml) (white bars), anti-KDR antibodies (200 μg/ml) (grey bar) or recombinant $VEGF_{165}$ (3 μg/ml) (crossed bar). (B) PAEC/KDRs were incubated with bTat in the in the absence (−) or in the presence of the indicated GAGs (3 μg/ml). Then, cells were washed with PBS containing 2 M NaCl and the amount of cell-associated bTat was measured. Non specific binding was measured in the presence of suramin [5]. In panel B data are expressed as percent of bTat bound to cells in the absence of GAGs. Each bar represents the mean ± S.E.M. of three determinations in duplicate. Student's *t*-test: **, $P < 0.01$.

derivatives were then evaluated for their ability to inhibit xcTat/KDR interaction in PAEC/KDR cells. When tested at 3 μg/ml, only K5-OS(H) and K5-N,OS(H) prevented the interaction of Tat with PAEC/KDR cells. Under the same experimental conditions, K5-N,OS(L) and heparin did not exert any inhibitory effect (Fig. 4B) despite their ability to bind to Tat (see above).

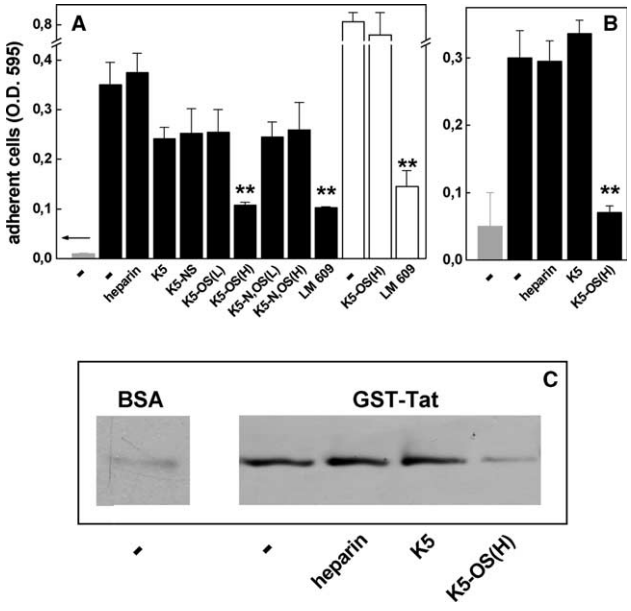

Fig. 5. Effect of K5 derivatives on Tat/$\alpha_v\beta_3$ interaction. GM 7373 cells (panel A) or HUVE cells (panel B) were allowed to adhere onto non tissue culture plates coated with BSA (grey bar), GST–Tat (black bars) or VN (white bars) (all at 20 μg/ml) in the absence (−) or in the presence of the indicated GAGs (1.5 μg/ml) or of the anti-$\alpha_v\beta_3$ monoclonal antibody LM609 (100 μg/ml). The number of adherent cells were then evaluated. Arrow: cells adherent to wells coated with GST protein (20 μg/ml). Each bar represents the mean ± S.E.M. of 3–6 determinations in duplicate. Student's *t*-test: **, $P < 0.01$. (C) Purified human $\alpha_v\beta_3$ was incubated onto wells coated with BSA or GST–Tat in the absence (−) or in the presence of the indicated GAGs (300 μg/ml). Then, plastic-bound proteins were extracted and analyzed by Western blotting with anti-β_3 antibodies.

3.4. Effect of K5 derivatives on Tat/$\alpha_v\beta_3$ integrin interaction

xcTat interacts with $\alpha_v\beta_3$ integrin receptors present on ECs [2,9]. Endothelial GM 7373 cells express $\alpha_v\beta_3$ integrin [21] and adhere to immobilized GST–Tat, but not to BSA or GST (Fig. 5A), in a $\alpha_v\beta_3$-dependent manner, as demonstrated by the inhibition exerted by the monoclonal anti-$\alpha_v\beta_3$ LM609 antibody (Fig. 5A). K5 derivatives were then evaluated for their ability to affect GM 7373 cell adhesion to immobilized GST–Tat. When tested at 1.5 μg/ml, only K5-OS(H) prevented the adhesion of ECs to GST–Tat-coated plastic. Again, despite their Tat-binding capacity, K5-N,OS(L), K5-N,OS(H) and heparin did not exerted any inhibitory effect when tested under the same experimental conditions (Fig. 5A). Also, K5-OS(H) did not inhibit the $\alpha_v\beta_3$-mediated GM 7373 cell adhesion to vitronectin (VN) (Fig. 5A), thus supporting the specificity of its Tat-antagonist activity. Finally, the $\alpha_v\beta_3$-antagonist activity of K5-OS(H) was not restricted to GM 7373 cells, as demonstrated by its capacity to inhibit also HUVE cell adhesion to immobilized Tat (Fig. 5B).

Purified $\alpha_v\beta_3$ integrin binds to immobilized GST–Tat (Fig. 5C). Specificity of the interaction is demonstrated by the lack of binding of $\alpha_v\beta_3$ to immobilized BSA. Preincubation of

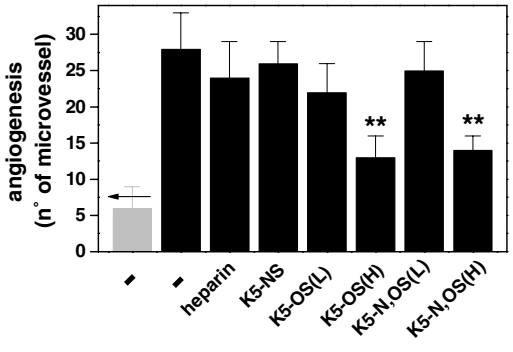

Fig. 6. Effect of K5 derivatives on Tat-induced angiogenesis in the chick embryo CAM. CAMs were implanted with gelatin sponges adsorbed with vehicle (grey bar) or with GST–Tat (400 ng) (black bars) in the absence (−) or in the presence of the indicated GAGs (50 μg). At day 12 the angiogenic response was scored as described [22]. Arrow: basal angiogenic response in CAMs treated with 400 ng of GST protein. Each bar represents the mean ± S.E.M. of three independent experiments. Student's *t*-test: **, $P < 0.01$.

immobilized GST–Tat with K5-OS(H) prevents the binding of $\alpha_v\beta_3$ whereas unmodified K5 and heparin were ineffective (Fig. 5C). Taken together, the data demonstrate that K5-OS(H) inhibits EC adhesion to immobilized Tat by preventing $\alpha_v\beta_3$ integrin interaction.

3.5. K5 derivatives inhibit the angiogenic activity of Tat protein

Tat protein exerts a potent angiogenic response in vivo by engaging both KDR and $\alpha_v\beta_3$ integrin receptors [2] (and references therein). Since K5-OS(H) and K5-N,OS(H) interfere with these interactions, they were evaluated for their ability to inhibit Tat-induced neovascularization in the CAM assay [22]. Both K5-OS(H) and K5-N,OS(H) exerted a significant inhibition whereas heparin, unmodified K5, and the other K5 derivatives tested were ineffective (Fig. 6).

4. Discussion

HIV-1 Tat, originally viewed as a viral transactivating factor with an intracellular mechanism of action, can act also as an extracellular growth factor endowed with heparin-binding capacity. On this basis, polyanionic compounds have been assessed as potential xcTat antagonists [3].

In the present paper, we evaluated the Tat-binding and antagonist capacity of a series of K5 derivatives with different sulfation patterns. A complete *N*-sulfation of glucosamine (GlcN) residues characterized the *N*- and *N*,*O*-sulfated derivatives whereas *O*-sulfation resulted in the almost complete 6-*O*-sulfation of GlcN residues in all the *O*- and *N, O*-sulfated derivatives that differed therefore for the extent of *O*-sulfation in the other positions [12].

Sulfated K5 derivatives act as xcTat-antagonists by mimicking heparin. Indeed, defined sulfated K5 derivatives interact with Tat, compete with ^{3}H-heparin for the binding to immobilized Tat, and prevent Tat interaction with cell-surface HSPGs.

When compared to heparin, K5-N,OS(H) displays a similar binding affinity (see Table 1) but a higher binding capacity [60 and 250 RU bound to the Tat surface, respectively, Fig. 1], despite their similar size. Previous observations had shown that a single heparin chain is able to bind up to 6 molecules of Tat with a cooperative interaction [6]. Thus, the different binding capacity between K5-N,OS(H) and heparin may be explained by the possibility that the highly sulfated, virtually homogeneous K5-N,OS(H) chain contains an even higher number of high affinity binding sites than the more heterogeneous heparin chain. Further studies are required to clarify this point.

Previous studies demonstrated that Tat/heparin interaction requires at least some 2-*O*-, 6-*O*-, and *N*-positions to be sulfated [7]. Non-sulfated K5 is devoid of any significant Tat-binding capacity, thus indicating that sulfate groups are essential also for K5 derivative interaction with Tat. Accordingly, only K5-OS(H) and K5-N,OS(H) [SO_3^-/COO^- equal to 3.8 for both compounds], and K5-N,OS(L) [SO_3^-/COO^- equal to 1.7] interact with Tat with high affinity. Among the Tat-binding compounds, K5-OS(H) consists of the virtually homogeneous repeat of GlcA2,3SO_3^-–GlcNAc3,6SO_3^- disaccharide units and most of K5-N,OS(H) sequences are represented by GlcA2,3SO_3^-–GlcNSO_3^-,6SO_3^-, whereas K5-N,OS(L) is mainly formed by GlcA-GlcNSO_3^-,6SO_3^- disaccharide units [12]. These data indicate that both the degree of sulfation and charge distribution modulate the Tat-binding capacity of sulfated K5 derivatives completely composed of GlcA–GlcN sequences.

The capacity of K5-N,OS(L) to bind Tat protein indicates that disulfated GlcNSO_3^-,6SO_3^- residues alternated with non-sulfated GlcA residues are sufficient for a significant interaction. This observation, together with the lack of binding capacity of K5-NS and K5-OS(L) derivatives (consisting mostly of the homogenous repeats of GlcA–GlcNSO_3^- and GlcA–GlcNAc6SO_3^- disaccharide units, respectively), indicate that in the case of lower sulfated compounds the position of the sulfate groups are important, being both *N*-sulfated and 6-*O*-sulfated residues necessary for the interaction. The presence of additional sulfated groups in GlcA residues, as it occurs in K5-N,OS(H), does not seem to affect the association/dissociation rates of the interaction, even though it exerts a dramatic effect on some of the Tat-antagonist activities of the derivative in vitro and in vivo.

Interaction of xcTat with cell-associated HSPGs is required for its internalization and transactivation [4]. In agreement with their heparin-mimicking, Tat-binding capacity, K5-OS(H), K5-N,OS(L), and K5-N,OS(H) prevented Tat uptake and HIV-LTR transactivation in HL3T1 cells whereas K5, K5-NS, and K5-OS(L) were ineffective.

However, xcTat exerts angiogenic activity in vivo that reflects its capacity to interact with KDR and $\alpha_v\beta_3$ integrin receptors on EC-surface [2]. Different regions of the Tat protein appear to be involved in these interactions. The integrin recognition motif Arg–Gly–Asp is present in Tat and mediates $\alpha_v\beta_3$ interaction [9] without playing any role in heparin/HSPG [5] or KDR [8] interactions. In contrast, the heparin-binding, basic domain of Tat contributes significantly to integrin [23] and KDR [8] interaction.

Here, we demonstrate that K5-OS(H) and K5-N,OS(H) prevent the interaction of Tat with KDR. At variance, K5-OS(H), but not K5-N,OS(H), inhibits the interaction of Tat with $\alpha_v\beta_3$. In agreement with the tight cross-talk between tyrosine kinase angiogenic growth factor receptors and integrins in intracellular signaling and angiogenesis, both K5 derivatives suppressed the angiogenic activity of Tat. Interestingly, low sulfated K5-N,OS(L) and heparin were unable to affect KDR and $\alpha_v\beta_3$ interactions despite their capacity to bind Tat with high affinity. Consequently, they did not exert any anti-angiogenic activity in vivo.

Taken together, our data indicate that the capacity of K5 derivatives to bind Tat thus preventing its interaction with cell surface HSPGs is sufficient to affect its cell internalization and LTR-transactivating activity. However, this does not necessarily result in a significant Tat antagonist activity for different signaling receptors and for neovascularization. Also, the different capacity of K5-OS(H) and K5-N,OS(H) to affect $\alpha_v\beta_3$ and/or KDR interaction indicates the possibility to dissociate different Tat antagonist activities by tailoring the distribution of sulfate groups along the GAG chain. This dissociation may depend on distinct interactions of the various K5 derivatives with Tat protein that mask different functional domains and/or to a different capacity of K5 derivatives to bind directly to Tat receptors, as already demonstrated for the FGF-2/FGFR system [26]. Further studies with K5 derivatives may shed new lights on the mechanism(s) of action of xcTat.

Heparin binding to Tat is size dependent, being 5–6 saccharide residues the minimal structure required for a low af-

finity interaction [6]. On the other hand, the reduction in size of a GAG ameliorates its pharmacokinetics and its bioavailability in the blood stream [27]. Relevant to this point, we observed that a low molecular weight K5-N,OS(H) (4.8 kDa) binds GST–Tat and inhibits its transactivating activity with affinity/potency that are comparable to those of the parent compound (data not shown), thus suggesting the possibility to reduce the size of K5 derivatives without losing their Tat-antagonist activity. Also, K5-OS(H) and K5-N,OS(H) are endowed with low anti-coagulant activity when compared to heparin (Oreste P. and Zoppetti G., unpublished observation).

xcTat contributes to HIV replication associated with primary HIV infection, when synchronized virion replication takes place [28]. K5-OS(H) and K5-N,OS(H) inhibit HIV replication by preventing virion attachment and/or entry in T cells and macrophages [14]. Also, K5-N,OS(H) is a potent antagonist of FGF-2 [12] that contributes to the progression of Kaposi's sarcoma, an ipervascularized lesion frequently found in male AIDS patients.

These observations, together with the results here reported, suggest that sulfated K5 derivatives can be exploited for the design of multi-target drugs able to prevent/retard HIV-infection/AIDS progression and to cure AIDS-associated pathologies, including Kaposi's sarcoma.

Acknowledgements: We thank Dr. B. Musulin for cell adhesion experiments. This work was supported by grants from ISS (AIDS Project) and MIUR (Cofin 2003) to M.R., from European Community (''Biologically Active Novel Glycosaminoglycans'' contract no. QLK3-CT-1999-00536), AIRC and MIUR (Centro di Eccellenza ''IDET'', FIRB, Cofin 2002) to M.P.

References

[1] Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R.A., Wingfield, P. and Gallo, R.C. (1993) J. Virol. 67, 277–287.
[2] Rusnati, M. and Presta, M. (2002) Angiogenesis 5, 141–151.
[3] Rusnati, M. and Presta, M. (2002) Drug Fut. 27, 481–493.
[4] Tyagi, M., Rusnati, M., Presta, M. and Giacca, M. (2001) J. Biol. Chem. 276, 3254–3261.
[5] Rusnati, M., Tulipano, G., Urbinati, C., Tanghetti, E., Giuliani, R., Giacca, M., Ciomei, M., Corallini, A. and Presta, M. (1998) J. Biol. Chem. 273, 16027–16037.
[6] Rusnati, M., Tulipano, G., Spillmann, D., Tanghetti, E., Oreste, P., Zoppetti, G., Giacca, M. and Presta, M. (1999) J. Biol. Chem. 274, 28198–28205.
[7] Rusnati, M., Coltrini, D., Oreste, P., Zoppetti, G., Albini, A., Noonan, D., d'Adda di Fagagna, F., Giacca, M. and Presta, M. (1997) J. Biol. Chem. 272, 11313–11320.
[8] Albini, A., Soldi, R., Giunciuglio, D., Giraudo, E., Benelli, R., Primo, L., Noonan, D., Salio, M., Camussi, G., Rockl, W. and Bussolino, F. (1996) Nat. Med. 2, 1371–1375.
[9] Barillari, G., Gendelman, R., Gallo, R.C. and Ensoli, B. (1993) Proc. Natl. Acad. Sci. USA 90, 7941–7945.
[10] Vann, W.F., Schmidt, M.A., Jann, B. and Jann, K. (1981) Eur. J. Biochem. 116, 359–364.
[11] Casu, B., Grazioli, G., Razi, N., Guerrini, M., Naggi, A., Torri, G., Oreste, P., Tursi, F., Zoppetti, G. and Lindahl, U. (1994) Carbohydr. Res. 263, 271–284.
[12] Leali, D., Belleri, M., Urbinati, C., Coltrini, D., Oreste, P., Zoppetti, G., Ribatti, D., Rusnati, M. and Presta, M. (2001) J. Biol. Chem. 276, 37900–37908.
[13] Borgenstrom, M., Jalkanen, M. and Salmivirta, M. (2003) J. Biol. Chem. 278, 49882–49889.
[14] Vicenzi, E., Gatti, A., Ghezzi, S., Oreste, P., Zoppetti, G. and Poli, G. (2003) Aids 17, 177–181.
[15] Rusnati, M., Urbinati, C., Caputo, A., Possati, L., Lortat-Jacob, H., Giacca, M., Ribatti, D. and Presta, M. (2001) J. Biol. Chem. 276, 22420–22425.
[16] Hatton, M.W., Berry, L.R., Machovich, R. and Regoeczi, E. (1980) Anal. Biochem. 106, 417–426.
[17] Esko, J.D. (1991) Curr. Opin. Cell Biol. 3, 805–816.
[18] Wright, C.M., Felber, B.K., Paskalis, H. and Pavlakis, G.N. (1986) Science 234, 988–992.
[19] Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C.H. (1994) J. Biol. Chem. 269, 26988–26995.
[20] Grinspan, J.B., Mueller, S.N. and Levine, E.M. (1983) J. Cell Physiol. 114, 328–338.
[21] Rusnati, M., Tanghetti, E., Dell'Era, P., Gualandris, A. and Presta, M. (1997) Mol. Biol. Cell 8, 2449–2461.
[22] Ribatti, D., Gualandris, A., Bastaki, M., Vacca, A., Iurlaro, M., Roncali, L. and Presta, M. (1997) J. Vasc. Res. 34, 455–463.
[23] Mitola, S., Soldi, R., Zanon, I., Barra, L., Gutierrez, M.I., Berkhout, B., Giacca, M. and Bussolino, F. (2000) J. Virol. 74, 344–353.
[24] Moscatelli, D. (1987) J. Cell. Physiol. 131, 123–130.
[25] Arese, M., Ferrandi, C., Primo, L., Camussi, G. and Bussolino, F. (2001) J. Immunol. 166, 1380–1388.
[26] Coltrini, D., Rusnati, M., Zoppetti, G., Oreste, P., Grazioli, G., Naggi, A. and Presta, M. (1994) Biochem. J. 303 (Pt 2), 583–590.
[27] Bendetowicz, A.V., Beguin, S., Caplain, H. and Hemker, H.C. (1994) Thromb. Haemost. 71, 305–313.
[28] Peterlin, B.M., Adams, M., Alonso, A., Baur, A., Ghosh, S., Lu, X. and Luo, X. (1993) in: Human Retroviruses (Cullen, B.R., Ed.), pp. 75–100, Oxford University Press, Oxford.
[29] Khalifa, M.B., Choulier, L., Lortat-Jacob, H., Altschuh, D. and Vernet, T. (2001) Anal. Biochem. 293, 194–203.
[30] Delehedde, M., Lyon, M., Gallagher, J.T., Rudland, P.S. and Fernig, D.G. (2002) Biochem. J. 366, 235–244.