Single chain fragment anti-heparan sulfate antibody targets the polyamine transport system and attenuates polyamine-dependent cell proliferation

JOHANNA E. WELCH[1], PER BENGTSON[1], KATRIN SVENSSON[1], ANDERS WITTRUP[1], GUIDO J. JENNISKENS[2], GERDY B. TEN DAM[2], TOIN H. VAN KUPPEVELT[2] and MATTIAS BELTING[1]

[1]Department of Clinical Sciences, Division of Oncology, Lund University, Barngatan 2:1, SE-22185, Lund, Sweden; [2]Department of Biochemistry, Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, P.O. Box 9101, 6500 HB, Nijmegen, The Netherlands

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Abstract. The growth-promoting polyamines are polybasic compounds that efficiently enter cancer cells by as yet incompletely defined mechanisms. Strategies to inhibit their internalization may have important implications in the management of tumor disease. Here, we show that cellular binding and uptake of polyamines are inhibited by a single chain variable fragment anti-heparan sulfate (HS) antibody. Polyamine uptake was inhibited in a dose-dependent manner, and was associated with compensatory up-regulation of ornithine decarboxylase (ODC), i.e. the key enzyme of the polyamine biosynthesis pathway. Conversely, depletion of intracellular polyamines by the specific ODC-inhibitor α-difluoromethylornithine (DFMO) resulted in increased cellular binding of polyamine and anti-HS antibody. Importantly, anti-HS antibody also efficiently targeted DFMO-induced polyamine uptake, and combined polyamine biosynthesis inhibition by DFMO, and uptake inhibition by anti-HS antibody attenuated tumor cell proliferation *in vitro*. In conclusion, cell-surface HS proteoglycan is a relevant target for antibody-mediated inhibition of the uptake of polyamines, and polyamine-dependent cell proliferation.

Correspondence to: Dr Mattias Belting, Department of Clinical Sciences, Section of Oncology, Lund University, Barngatan 2:1, SE-221 85, Lund, Sweden
E-mail: mattias.belting@med.lu.se

Abbreviations: DFMO, alpha-difluoromethylornithine; HSPGs, heparan sulfate proteoglycans; HIV-Tat, human immunodeficiency virus transactivating protein; ODC, ornithine decarboxylase; scFv, single chain variable fragment

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Introduction

The polyamines (putrescine, spermidine, and spermine) are essential for cell proliferation, and increased polyamine levels have been associated with cell transformation and cancer disease (1-3). Depletion of intracellular polyamines by α-difluoromethylornithine (DFMO), i.e. a specific inhibitor of polyamine biosynthesis, results in efficient growth-inhibition of tumor cells *in vitro*, but the outcome of DFMO in anti-cancer trials has been overall disappointing due to compensatory recruitment of circulating polyamines *in vivo* (1-3). Anti-tumor therapy directed at the polyamine system thus requires simultaneous targeting of polyamine biosynthesis and uptake.

It is well established that a wide variety of tumor cells efficiently internalize polyamines; however, the molecular details of the polyamine uptake pathway/s remain to be defined. Previous studies suggest an involvement of cell-surface heparan sulfate proteoglycans (HSPGs) (4-7) that constitute a family of polysaccharide-substituted proteins involved in the binding of protease inhibitors, lipoproteins, growth factors, and viruses with implications for cardiovascular and infectious diseases and cancer (6,8-10). HS constitutes a linear polysaccharide of repetitive glucuronic acid/N-acetylglucosamine disaccharide units, which are modified to varying degrees by epimerization of glucuronic acid into iduronic acid and sulfation at various positions along the chain, resulting in a complex and highly negatively charged polymer structure (8-10). Cell-surface HSPGs thus form a polyanion envelope at the cell-matrix interface, allowing for electrostatic interactions with certain arginine/lysine-rich motifs of protein ligands (9,11).

Here, we have studied the effects of single chain variable fragment (scFv) anti-HS antibodies on the uptake of polyamines, and polyamine-dependent cell-growth. The anti-HS antibody RB4EA12 inhibits polyamine binding and internalization as well as polyamine-dependent proliferation in several types of cancer cells, i.e. A549 human lung carcinoma, HeLa human cervix adenocarcinoma, and CHO cells. The results point at a role for anti-HS antibodies as a novel strategy

for manipulation of the polyamine system. The fact that anti-HS antibody efficiently attenuates cell-growth dependent on the uptake of extracellular polyamines, implicates the HSPG-mediated uptake pathway as a relevant target for antibody-based anti-tumor therapy. Moreover, to our knowledge, this is the first study to demonstrate inhibition of HSPG function by recombinant scFv antibody.

Materials and methods

Materials. Polyamines and fine grade chemicals were from Sigma Chemical Co., and [^{14}C]polyamines were from Amersham International, UK. HIV-Tat peptide (GRKKRR QRRRPPQC) labelled at the C-terminus with AlexaFluor-647, was synthesized by Innovagen AB, Lund, Sweden. Fluorophores and secondary antibodies were from Invitrogen. DFMO was from Ilex Oncology, USA. Cell media and supplements were from Sigma, Sweden. scFV antibodies directed against HS were obtained by biopanning against HS isolated from bovine kidney (HS4E4) (12), and from skeletal muscle from mouse (AO4B08) and human (RB4EA12) (13). Large scale antibody production was performed as previously described (12).

Cell culture. Wild-type Chinese hamster ovary (CHO)-K1 cells, PG-deficient pgsA-745, HSPG-deficient pgsD-677 CHO cell mutants (14), human cervix adenocarcinoma (HeLa) and lung carcinoma (A549) cells were from the American Type Culture Collection (ATCC). CHO cells deficient in ornithine decarboxylase (ODC) activity (15), denoted CHO ODC- were kindly provided by Dr Lo Persson, Lund University, Sweden. Wild-type and CHO ODC- cells were cultured in F12K, and 50% DMEM/50% F12K nutrient mixture supplemented with 100 μM putrescine, respectively. HeLa was cultured in DMEM nutrient mixture and A549 in F12K supplemented with 1% non-essential amino acids. Routine culture was performed in a humidified 5% CO_2 incubator at 37˚C, using the respective media supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (growth medium).

scFv anti-HS antibody binding. CHO-K1, pgsA-745 or pgsD-677 cells were detached with concentrated PBS (2X) supplemented with 0.5 mM EDTA, washed with PBS 1% BSA (w/v), and incubated with anti-HS scFv antibody RB4EA12 (titre 1:5) in PBS 1% BSA for 30 min on ice. Cells were washed in PBS 1% BSA and incubated with mouse anti-VSV antibody (1:100, Sigma), recognizing the VSV-epitope of anti-HS antibody, for 30 min on ice, followed by rinsing in PBS 1% BSA and incubation with goat anti-mouse Alexa Fluor-488 conjugated antibody (1:200, Invitrogen) for 30 min on ice. Finally, cells were washed in PBS 1% BSA and analyzed by flow cytometry on a FACS-Calibur instrument integrated with Cell-Quest software (BD Biosciences). HeLa cells were grown in absence or presence of 5 mM DFMO for 72 h. Cells were incubated with anti-HS scFv antibody RB4EA12 (titre 1:20), mouse anti-VSV antibody (1:500, Sigma) and goat anti-mouse Alexa Fluor-488 conjugated antibody (1:200, Invitrogen) as described above. Cells were washed in PBS 1% BSA and analyzed by flow cytometry on a FACS-Calibur instrument integrated with Cell-Quest software (BD Biosciences).

Polyamine binding and uptake. For polyamine uptake and binding experiments, cells were plated in 48-well culture plates and 24-well culture plates at 25,000 cells/well and 50,000 cells/well, respectively in growth medium, with or without 5 mM DFMO. Cells were grown for three days, after which varying concentrations of polyamines and [^{14}C]polyamines, in the presence or absence of different titres of anti-HS antibody, were added in serum-free medium. Cells were incubated for 20 min at 4˚C or 37˚C for polyamine binding ([^{14}C]-specific activity of 93 Ci/mol) and uptake experiments ([^{14}C]-specific activity of 31 Ci/mol), respectively. The incubation medium was removed and cells were extensively rinsed with cold F12K (binding experiments) or F12K containing 1 mM unlabeled polyamine (uptake experiments), followed by cell lysis in 0.5 M NaOH for 1 h at 37˚C. An aliquot of the cell lysate was neutralized with 0.5 M HCl and then analyzed for [^{14}C]polyamine content by scintillation counting (Beckman Counter).

HIV-Tat peptide uptake. AlexaFluor-647-labeled Tat (Tat-AF647) peptide (10 μg/ml), in the presence or absence of different titres (1:80-1:5) of anti-HS antibodies, were added to extensively rinsed, subconfluent CHO cells in 48-well culture plates at 37˚C for 30 min. To exclude the presence of cell-surface associated ligand, cells were trypsinized and extensively rinsed with PBS 1% BSA. Uptake of Tat-AF647 peptide was determined by flow cytometry on a FACS-Calibur instrument integrated with Cell-Quest software (BD Biosciences).

Confocal fluorescence microscopy. scFV antibody RB4EA12 (titre 1:40), mouse anti-VSV antibody (1:500) and goat anti-mouse-Alexa Fluor 488 antibody (1:100) were pre-incubated in serum free medium at 4˚C for 30 min to allow for complex formation. Subconfluent wild-type, pgsA-745 and pgsD-677 CHO cells in 8 well chamber slides were rinsed with PBS and incubated with antibody complexes at 4˚C for 30 min followed by three consecutive rinses with ice-cold PBS 1% BSA and fixation with 4% (w/v) paraformaldehyde. Cells were counter-stained for f-actin using phalloidin-Alexa 543 (1:100), and for nuclei using topro (1:100), followed by mounting in Permafluor (Beckman Coulter). Cells were analyzed using Leica confocal scanning equipment TCS SP2 II with a 63x immersion oil objective.

Immunoblot assays. Cells were seeded in 12-well plates at 40,000 cells/well and allowed to adhere and grow for 72 h. The cells were incubated with varying titres (1:10 and 1:5) of RB4EA12 for 8 h and subsequently washed with PBS, and lysed in radioimmune precipitation lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) supplemented with complete mini protease inhibitor cocktail (Roche Diagnistics GmbH, Germany). The insoluble pellet was discarded and protein concentration was determined using BCA™ Protein Assay Kit (Pierce). Equal amounts of protein were mixed with 4x NuPage LDS sample buffer (Invitrogen) and NuPage

Table I. scFV anti-HS antibodies used in this study.

Clone	CDR3 sequence	Preferred HS modifications	Ref.
AO4B08	SLRMNGWRAHQ	GlcNS, IdoA, 2OS, 6OS	(16)
RB4EA12	RRYALDY	GlcNAc, GlcNS, 6OS	(13[a])
HS4E4	HAPLRNTRTNT	GlcNAc, GlcNS, IdoA	(16)

Given are the amino acid sequences for the VH complementary determining region (CDR)-3 and preferred HS structures. [a]Van Kuppevelt, unpublished data.

sample reducing agent (Invitrogen), heated at 70˚C for 10 min and fractionated by electrophoresis in a 4-12% polyacrylamide-SDS gel. The resolved proteins were electroblotted to PVDF membrane (Pall Corp.). The resulting blots were incubated with rabbit polyclonal anti-ODC (kindly provided by Dr L. Persson) (1:7000) and rabbit polyclonal anti-ß-actin (Abcam) (1:8000) followed by horseradish peroxidase-conjugated IgG antibodies. Signals were developed using ECL Western blotting substrate (Pierce) at 25˚C and the membranes were exposed to X-ray film (Hyperfilm MP, Amersham). Band intensities were quantified by densitometry using TotalLab v1.11 software and ODC levels were normalized to ß-actin.

Cell proliferation assay. A549, HeLa, CHO wild-type and CHO ODC- cells were seeded into 48-well culture plates at 5,000 (A549) or 2,000 (HeLa, CHO) cells/well. CHO ODC- cells, which are dependent on exogenous polyamines for normal growth, were cultured in growth medium in the presence or absence of 1 μM spermine and RB4EA12 at varying titres (1:10 and 1:5) for 72 h. HeLa was grown in serum-free conditions in the absence or presence of 5 mM DFMO with or without the addition of 1 μM spermine and RB4EA12 (titre 1:10) for 72 h. A549 was grown in growth medium in the presence or absence of 5 mM DFMO with or without the addition of 1 μM spermine and RB4EA12 (titre 1:20) for 72 h. As a control, RB4EA12 (titre 1:20) alone was added to control cells (no DFMO treatment). The cells were rinsed with PBS, released by trypsinization and the number of viable cells was determined by trypan blue exclusion. The [^{3}H]thymidine incorporation assay was employed to assess DNA synthesis. Wild-type CHO cells were grown in serum-free conditions in the presence or absence of 5 mM DFMO with or without the addition of 0.1 μM spermine and AO4B08 (titre 1:10) or RB4EA12 (titre 1:10) for 48 h. As a control, RB4EA12 (titre 1:10) antibody alone was added to control cells (no DFMO treatment). The growth medium was changed to serum free F12K; DFMO, spermine and scFv anti-HS antibodies were added simultaneously with 3 μCi/ml [^{3}H]thymidine and the cells were allowed to proliferate an additional 20 h. The cells were then washed extensively with PBS, and cellular proteins were precipitated with 10% cold trichloroacetic acid for 30 min. After extensive washing with PBS, DNA from cell extracts was obtained by incubation with 0.1 M NaOH for 5 min at room temperature. An aliquot of the cell lysate was neutralized with 0.5 M HCl and the amount of incorporated [^{3}H]thymidine was determined by liquid scintillation.

Cell viability assay. Annexin V labelling was used to detect early apoptotic cells. A549 was seeded in 24-well plates at 10,000 cells/well and allowed to grow in growth medium in the presence or absence of 5 mM DFMO with or without the addition of 1 μM spermine and RB4EA12 (titre 1:20) for 72 h. As a control, RB4EA12 alone was added to control cells (no DFMO treatment). The cells were detached by trypsinization and labelled with Annexin V using TACS™ Annexin V-FITC kit (R&D Systems, Inc) as described by the manufacturer. Annexin V labelling was determined using flow cytometry on a FACS-Calibur instrument integrated with Cell-Quest software (BD Biosciences).

Statistical analyses. Confocal fluorescence microscopy, flow cytometry and cell proliferation figures are representative of at least two independent experiments. Data points in the polyamine binding and uptake experiments are the mean ± SD (n=5-6). Statistical significance was determined by Student's t-test.

Results

HSPG-specific binding of scFv antibody to live cells. The scFv antibodies used in this study were previously selected for HS specificity on surfaces coated with various polyanions, e.g. DNA, chondroitin sulfate and HS preparations from different tissues (13,16) (see Table I). In initial studies, HSPG-specific binding to live cells of the antibody RB4EA12 was evaluated quantitatively in wild-type and HS-deficient CHO cell mutants by flow cytometry analysis (Fig. 1). The binding specificity for cell-surface HS was demonstrated by the fact that antibody binding was substantially reduced in mutant cells (pgsA-745) that express ~5% of total PG as compared with wild-type cells (~6% binding as compared with wild-type cells) (Fig. 1B). The antibody showed no binding to mutant cells (pgsD-677) that are specifically defective in HSPG formation and that express about 3-fold more chondroitin sulfate PG than wild-type cells (Fig. 1C). Confocal fluorescence microscopy studies (Fig. 1D-F) showed multi-focal binding of anti-HS antibody at the cell-surface of wild-type cells, and a substantial amount of antibody appeared to be located to the filopodia (Fig. 1D, insert), whereas HSPG-

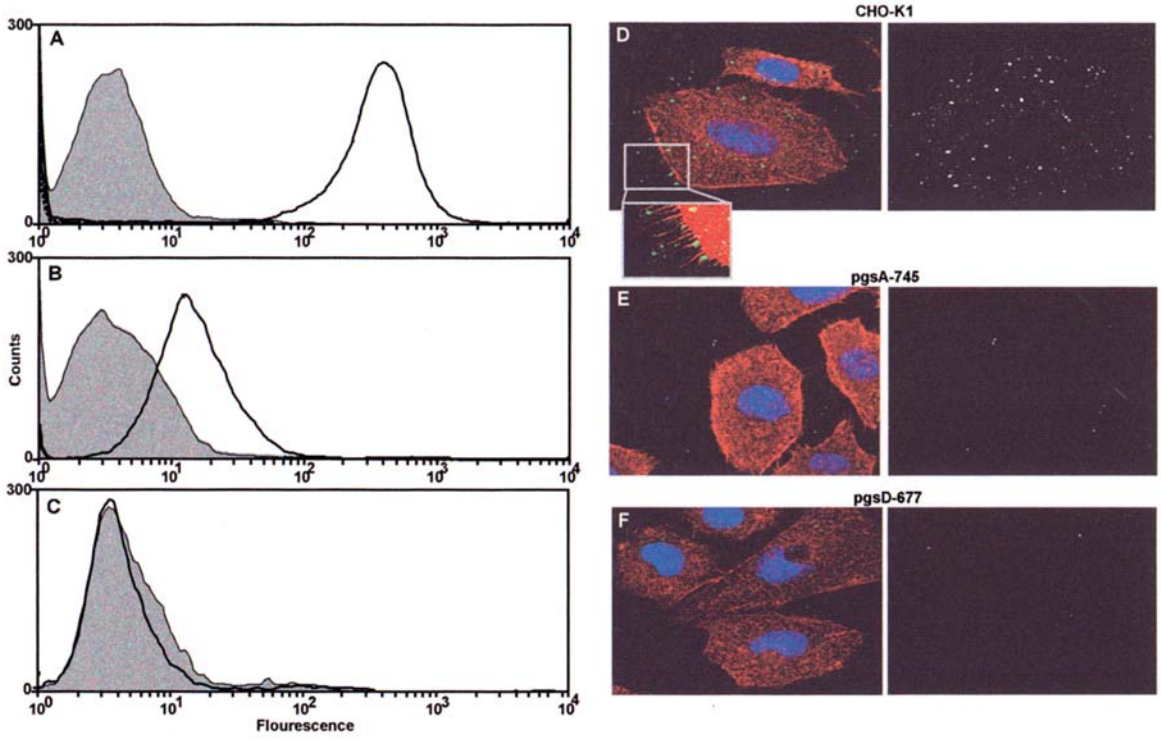

Figure 1. Characterization of RB4EA12 anti-HS antibody binding to cell-surface HSPGs. (A) Wild-type CHO cells (CHO-K1), (B) PG-deficient CHO cells (pgsA-745) and (C) CHO cells specifically devoid of HSPG (pgsD-677) were incubated with RB4EA12 (titre 1:5) at 4˚C for 30 min. As a control, the cells were incubated with secondary and tertiary antibodies alone (shaded area). Cellular binding of anti-HS antibodies was quantified by flow cytometry as described in Materials and methods. Please note logarithmic scale on the x-axis. (D-F) Left column, composite confocal fluorescence microscopy images showing HSPG-specific cell-surface binding of RB4EA12. (D) CHO-K1, (E) pgsA-745 and (F) pgsD-677 cells were incubated with RB4EA12, mouse anti-VSV, and goat anti-mouse-Alexa Flour 488, followed by counterstaining with phalloidin-Alexa Flour 543 (actin cytoskeleton) and Topro (nuclei). (D insert) Enhanced visualization of RB4EA12-binding to filopodia. (D-F) Right columns, greyscale images visualizing HSPG-specific cell-surface binding of RB4EA12. Cells incubated with mouse anti-VSV and goat anti-mouse-Alexa flour 488 alone were negative (results not shown). Results are representative of at least two independent experiments.

deficient cells were negative, confirming specific antibody binding to cell-surface HSPGs *in vitro*.

Effects of anti-HS antibodies on cellular binding and uptake of polyamines and HIV-Tat. We next investigated the effects of RB4EA12 on the internalization of the polyamines spermine and spermidine. As shown in Fig. 2, RB4EA12 dose-dependently inhibited the uptake of both spermine (Fig. 2A) and spermidine (Fig. 2B). The results correlated with dose-dependent cellular binding of scFv antibody in the same titre interval (Fig. 2B, insert). The effect on cellular binding of polyamines varied with the epitope specificity of different anti-HS antibodies (Fig. 2C; see also Table I) as suggested by the fact that two other anti-HS antibodies, i.e. AO4B08 and HS4E4, both showed limited inhibition (~15 and 5% respectively) compared to RB4EA12 (~60% inhibition). The effects on polyamine cell-surface binding correlated with effects on uptake, i.e. AO4B08 and HS4E4 were both inactive (data not shown).

To test the possibility that lack of inhibitory activity by other anti-HS antibodies was attributed to poor HS binding, we next studied the effects of anti-HS antibodies on the uptake of another well-known HSPG ligand, i.e. HIV-Tat transduction peptide. Polyamine binding to HS (K_d of ~37 μM) (17) is considerably weaker than the binding of HIV-Tat to HS (K_d in the low nM range) (18) and accordingly, the Tat peptide was recently shown to efficiently inhibit polyamine uptake (19). As shown in Fig. 2D, both RB4EA12 and AO4B08 inhibited Tat internalization in a dose-dependent and equipotent manner, with ~60% inhibition at an antibody titre of 1:5. Differential effects of the anti-HS antibodies on polyamine (efficient inhibition by RB4EA12 only) and Tat (equipotent inhibition by RB4EA12 and AO4B08) uptake suggest that the two ligands have different structural requirements for HSPG-dependent internalization, and that RB4EA12 competition with polyamines involves some degree of structural specificity.

RB4EA12 is a specific tool for manipulation of the polyamine system. To corroborate these findings, and to exclude cell-line specific effects, we next studied effects of RB4EA12 on polyamine uptake in human lung (A549, Fig. 3A) and cervix (HeLa, Fig. 3B) carcinoma cell lines. Polyamine uptake was inhibited dose-dependently (~60% inhibition at an antibody titre of 1:10) in both A549 and HeLa cells. It is well established that inhibition of polyamine biosynthesis is compensated for by increased polyamine internalization. Accordingly, treatment of A549 and HeLa cells with DFMO, i.e. a highly specific inhibitor of polyamine biosynthesis, increased polyamine uptake by ~4- and 1.6-fold in the respective cell-line. Importantly, also DFMO-induced uptake was inhibited by RB4EA12 in a dose-dependent manner (Fig. 3A and B,

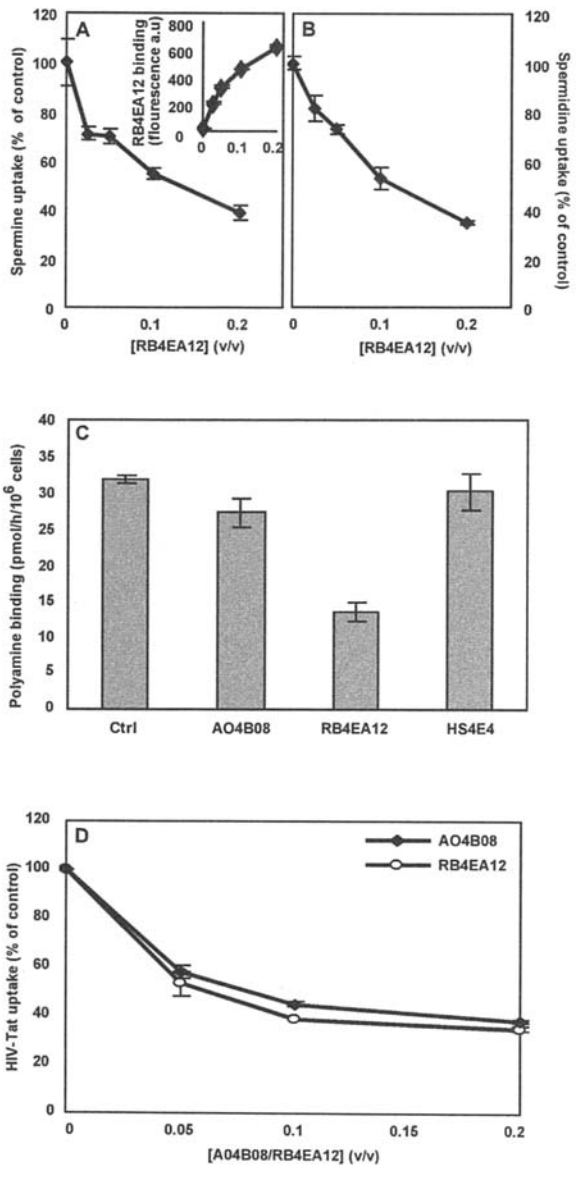

Figure 2. Effects of anti-HS antibodies on cellular binding and internalization of the HS-binding ligands polyamines and HIV-Tat. CHO cells were incubated with 0.5 μM [^{14}C]spermine (A and C) or 0.5 μM [^{14}C]spermidine (B) with or without RB4EA12, AO4B08 or HS4E4 at varying titres [1:40-1:5 in (A) and (B) and 1:5 in (C) for 20 min at 37˚C (A and B) or 4˚C (C), for polyamine uptake and binding experiments, respectively. (A, insert) Dose-dependent inhibition of polyamine uptake corresponds with dose-dependent cell-surface binding of RB4EA12, as determined by flow cytometry. Polyamine binding and uptake were determined by scintillation counting and results are presented as means ± SD (n=5-6). (D) CHO cells were incubated with HIV-TAT-Alexa Flour 647 (10 μg/ml) and AO4B08 or RB4EA12 at varying titres (1:20-1:5) at 37˚C for 30 min, and HIV-TAT uptake was determined by flow cytometry. Results are the mean ± SD (n=3).

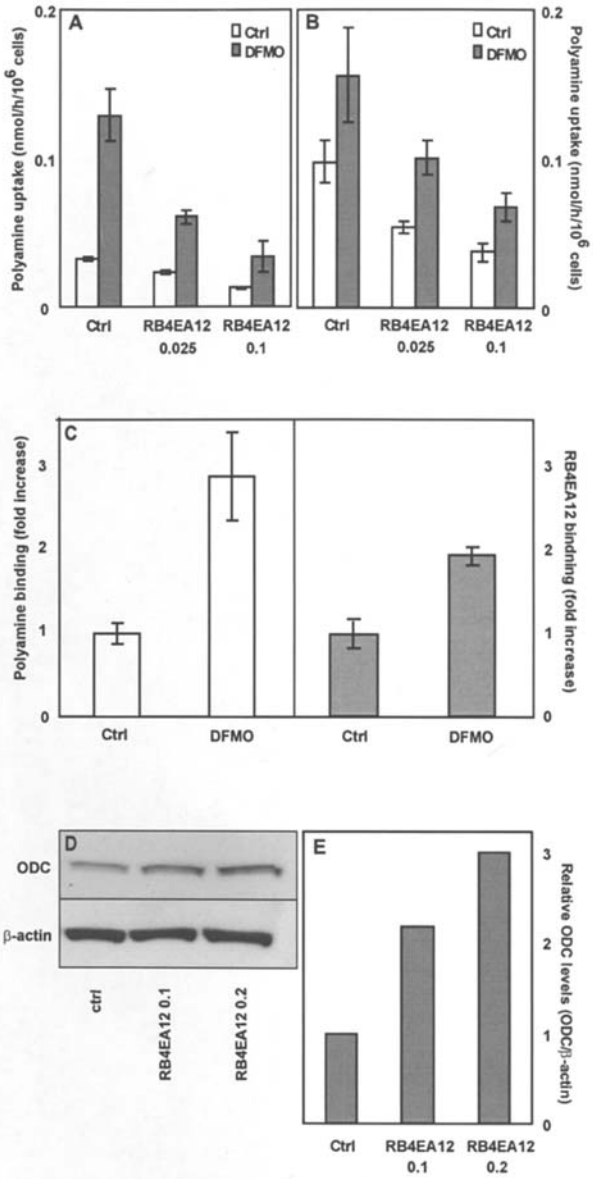

Figure 3. Increased binding of and efficient polyamine uptake inhibition by RB4EA12 in DFMO-treated human carcinoma cells. (A) A549 or (B) HeLa cells were grown in the absence or presence of 5 mM DFMO for three days and then incubated with 0.5 μM [^{14}C]spermine with or without RB4EA12 at varying titres (1:40 and 1:10) for 20 min at 37˚C. Polyamine uptake was determined by scintillation counting and results are mean ± SD (n=3) (C) HeLa cells were grown in the absence or presence of 5 mM DFMO for 3 days and then incubated with either 0.5 μM [^{14}C]spermine (white bars) or RB4EA12 (grey bars) (titre 1:20) at 4˚C for 20 and 30 min, respectively. Cells incubated with RB4EA12 were subsequently incubated with mouse anti-VSV followed by goat anti-mouse-Alexa fluor 488 at 4˚C as described in Materials and methods and cellular binding was determined by flow cytometry. Results are presented as fold increase as compared with control (no DFMO treatment) and are representative of two independent experiments (n=3). (D) Reciprocal induction of polyamine biosynthesis by RB4EA12: HeLa cells were incubated with RB4EA12 at varying titres (1:10 and 1:5) for 8 h. Cellular extracts were fractionated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-ODC antibody and anti-ß-actin as loading control. Results are representative of two independent experiments. (E) Band intensities were quantified by densitometry using TotalLab v1.11 software. ODC levels were normalized to ß-actin and presented as relative values.

filled bars). At the highest titer (1:10), uptake was reduced by ~75% and 60% in A549 and HeLa cells, respectively, as compared with untreated control.

Previous studies have shown that polyamine deprivation by DFMO treatment is associated with structural alterations of HS resulting in increased affinity for spermine-conjugated agarose (4). Therefore, it was of interest to test whether cellular binding of RB4EA12 is induced by DFMO treatment. As expected, treatment of HeLa cells with DFMO resulted in ~3-fold induction of cellular binding of polyamine (Fig. 3C, white bars). Interestingly, DFMO treatment resulted in ~2-fold induction of RB4EA12 cell-surface binding (Fig. 3C, grey bars). These data reinforce the notion that RB4EA12 and poly-

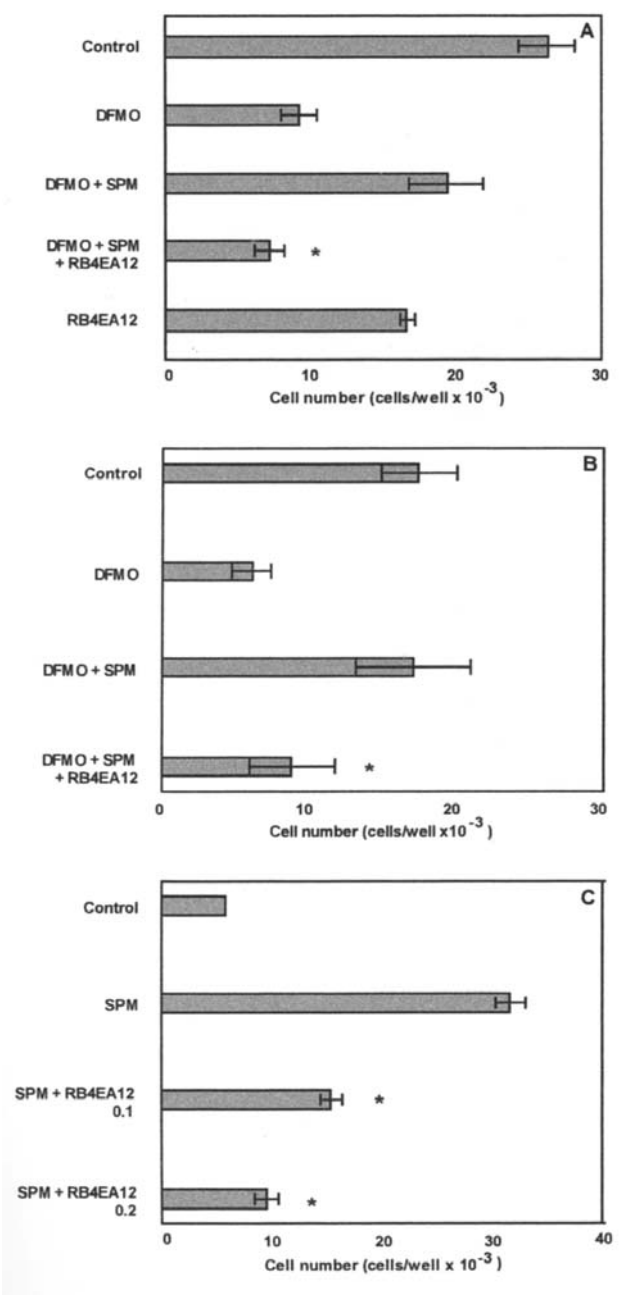

Figure 4. RB4EA12 inhibits polyamine dependent cell growth. (A) A549 or (B) HeLa cells were grown in the absence (control) or presence of DFMO with or without 1 μM spermine and RB4EA12 [titre 1:20 in (A) and 1:10 in (B)] for 72 h. As a control, RB4EA12 was added to control cells (titre 1:20) in (A). *P<0.02 vs. cells grown in the presence of DFMO and spermine (n=3-4). (C) CHO ODC- cells, which are dependent on exogenous polyamines for proliferation, were grown in the absence (control) or the presence of 1 μM spermine (SPM) with or without RB4EA12 at varying titres (1:5 and 1:10) for 72 h. *P<0.02 vs. cells grown in the presence of spermine only. (A-C) The number of viable cells was determined by trypan blue exclusion.

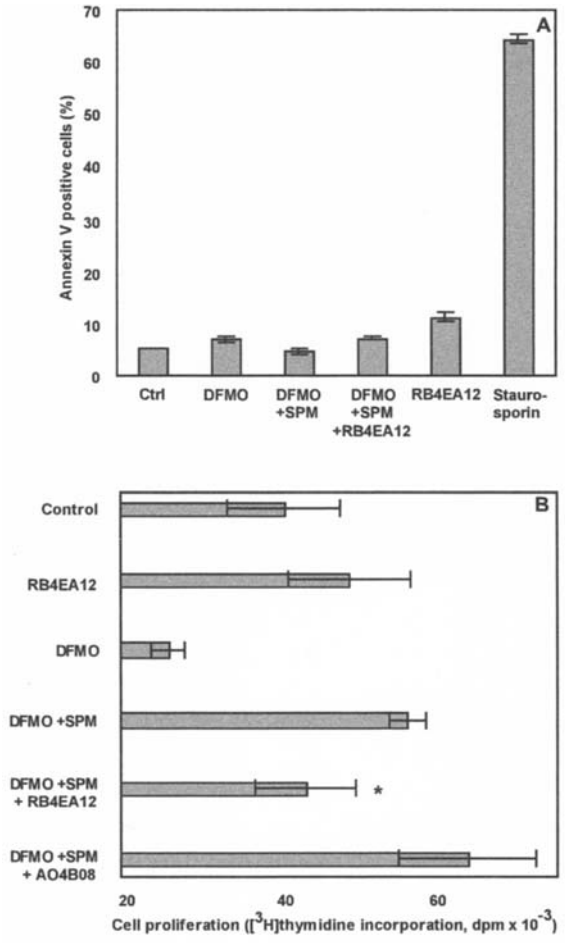

Figure 5. Combined treatment with DFMO and RB4EA12 has a cytostatic rather than cytotoxic effect on tumor cells *in vitro*. (A) A549 cells were grown in the absence (control) or presence of DFMO with or without 1 μM spermine and RB4EA12 (titre 1:20) for 72 h. As a control, RB4EA12 was added to control cells (titre 1:20). One μg/ml staurosporin was added to the cells as a positive control for apoptosis induction. Cells were released by trypsinization, washed and labelled with Annexin V. Annexin V labelling was analysed using flow cytometry. Results are representative of two independent experiments (n=3). (B) CHO cells were grown in the absence (control) or the presence of 5 mM DFMO (DFMO) with or without the addition of 0.1 μM spermine and RB4EA12 or AO4B08 (titre 1:10) for 48 h. As a control, RB4EA12 was added to control cells at a titre of 1:10. The medium was supplemented with 3 μCi/ml [^{3}H]thymidine and the cells were allowed to proliferate for another 24 h. DNA synthesis was determined by measurement of incorporated [^{3}H]thymidine. *P<0.05 vs. cells grown in the presence of DFMO and spermine (n=4).

amines share HSPG as interaction partner and that inhibition of polyamine biosynthesis is associated with increased expression of polyamine-binding HS at the cell-surface.

Polyamine homeostasis is tightly regulated and conditions that restrict the bioavailability of extracellular polyamine may induce the biosynthetic pathway. In order to study whether RB4EA12-mediated inhibition of polyamine uptake is biologically relevant, we next treated HeLa cells with RB4EA12 and analysed the expression level of ornithine decarboxylase (ODC), i.e. the key enzyme of polyamine biosynthesis. ODC levels were increased ~2- and 3-fold as compared with control at an antibody titre of 1:10 and 1:5, respectively (Fig. 3D and E). Compensatory up-regulation of the biosynthetic pathway is consistent with biologically relevant uptake inhibition by RB4EA12 antibody.

Inhibition of polyamine-dependent cell growth by RB4EA12. Depletion of intracellular polyamines by DFMO results in inhibition of tumor cell proliferation unless cells are provided with extracellular polyamines, which is the case in the *in vivo* situation (1-3). Accordingly, DFMO-treated A549 and HeLa cells are dependent on extracellular polyamine for efficient cell growth (Fig. 4A and B). We next investigated whether uptake-

inhibition by RB4EA12 results in attenuated proliferation under these conditions. Indeed, RB4EA12 counteracted the rescue by extracellular polyamine and significantly reduced polyamine dependent cell growth. Given that cell proliferation depends on several serum-resident HSPG-binding mitogenic factors, e.g. PDGF, FGFs, and VEGF, some cell-growth inhibition by antibody alone in control cells (no DFMO treatment) was not surprising (Fig. 4A).

To exclude the possibility that the observed effects on cell growth stem from unspecific effects of combined DFMO and anti-HS antibody treatment, we next performed similar experiments with CHO cell mutants genetically deficient in ODC activity (CHO ODC-). These cells are dependent on medium supplementation with extracellular polyamine for survival and growth (Fig. 4C). RB4EA12 significantly and dose-dependently inhibited polyamine-dependent cell-growth of CHO ODC- cells; at the higher titre (1:5) cell-growth was almost down to control levels.

Cytostatic effect of combined polyamine biosynthesis and uptake inhibition. Depletion of polyamines by biosynthesis inhibitors results in cell cycle arrest (2) and in some cases in apoptosis (20). To investigate whether the reduction of cell number by combined DFMO and anti-HS antibody treatment (Fig. 4) is a result of reduced cell proliferation or increased cell death, we performed cell labelling with Annexin V, i.e. a marker of early apoptotic events. The almost insignificant differences in the frequency of apoptotic cells (5% Annexin V positive cells in control and DFMO+SPM vs. 7% in DFMO and DFMO+SPM+RB4EA12) (Fig. 5A) could not explain the differences in cell numbers presented in Fig. 4A. Consistent with these results, RB4EA12 reduced polyamine-dependent DNA synthesis by ~40% (Fig. 5B). Notably, RB4EA12 alone had no effect on control cells (no DFMO treatment) under serum-free conditions. Taken together, these data suggest that the combined inhibition of polyamine synthesis by DFMO and uptake by anti-HS antibody has a cytostatic rather than cytotoxic effect on tumor cells *in vitro*.

Discussion

We have shown that the monoclonal, phage-derived anti-HS antibody RB4EA12 efficiently inhibits cellular binding and uptake of polyamines in several transformed cell-lines. This was true for constitutive as well as induced polyamine uptake upon polyamine biosynthesis inhibition. The polyamine system is intricately regulated with numerous feedback loops to ensure appropriate levels of intracellular polyamines (21). Polyamine depletion is associated with reduced cell proliferation, and under certain conditions increased apoptosis. Anti-HS antibody completely inhibited the proliferation of cells made dependent on extracellular polyamine by either genetic or pharmacological inhibition of ODC (Fig. 4). Inhibition of polyamine internalization by anti-HS antibody generated a compensatory up regulation of the biosynthesis machinery (Fig. 3D and E), providing further support for the notion that the HSPG-dependent uptake pathway is biologically relevant.

HIV-Tat has pleiotropic effects in AIDS pathology including activation of viral replication, stimulation of angiogenesis in Kaposi's sarcoma, and neural toxicity in late stage disease (22,23). The ability of HIV-Tat peptide to enter target cells is provided by an HS-binding, polybasic stretch of 13 amino acids (GRKKRRQRRRPPQ), and several reports have provided strong support for an HSPG-dependent uptake mechanism (6). Internalization of the HIV-Tat peptide was inhibited by both AO4B08 and RB4EA12 antibodies; however, inhibition of either polyamine or HIV-Tat uptake was incomplete (approximately 60% inhibition). Together with the fact that HIV-Tat peptide was previously shown to completely block polyamine uptake (19), this may indicate that Tat peptide is a more efficient competitor of HS binding than the studied scFv antibodies or, alternatively, that a non-HSPG dependent pathway for polyamine uptake is still sensitive to Tat inhibition. RB4EA12 reduction of polyamine uptake down to approximately 40% of control is in good agreement with previous studies demonstrating an approximately 50% reduction in spermine uptake in PG-deficient mutant cells and in Heparinase III treated human lung fibroblasts (4,5), which favours the latter hypothesis. The three anti-HS antibodies used in this study showed comparable levels of cell surface binding, and they all showed great HSPG specificity (data not shown); however, their ability to block the binding and internalization of polyamine differed considerably, suggesting epitope specific inhibition of polyamine internalization.

Our data show that the effects of anti-HS antibody on cell growth are associated with the inhibition of the polyamine uptake system; however, additional modes of action must be considered. Several serum-resident HSPG-interacting mitogens (e.g. VEGF, FGF-2, PDGF) may be susceptible to competition by anti-HS antibody and accordingly RB4EA12 also showed some growth-inhibitory effect in control cells stimulated by serum (Fig. 4A). Importantly, cell-growth dependent on extracellular polyamines was substantially more susceptible to RB4EA12 inhibition as compared with control cells, and RB4EA12 alone showed no inhibitory effects under serum-free conditions (Fig. 5B). Taken together, it is conceivable that the observed effects on cell growth stem from reduced bioavailability of extracellular polyamine and HS-binding growth factors in serum.

Given the important role for HSPG as co-receptors for several mitogens involved in tumorigenesis (24), several strategies to interfere with HSPG function have been developed and tested in experimental models. These include: HS mimetics, e.g. various heparins and dextran sulfate, and suramin, i.e. a polysulfated sulfonylurea; false substrates for HS biosynthesis, i.e. various small molecular weight hydrophobic compounds conjugated with xylose; and HS-degrading enzymes derived from bacteria. In this study, we have identified yet another strategy, i.e. the use of recombinant humanized anti-HS antibody that should be employed to target cell-surface HSPG function. Antibody-based therapy is one of the fastest growing therapy areas in clinical medicine, and antibodies targeting e.g. CD20, the epidermal growth factor receptor 2 (HER-2), VEGF, or EGF receptor have so far been approved in the treatment of aggressive B-cell lymphomas, breast cancer, kidney cancer, lung cancer, and colorectal cancer. The importance of HSPG for the growth-promoting activity of polyamines and a vast number of growth

factors makes HSPG an interesting target for antibody-based cancer therapy, and the results presented here warrant further studies to evaluate the anti-tumor effects of the combinational treatment with polyamine biosynthesis inhibitors *in vivo*.

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