

Fibroblast growth factor-2 antagonist activity and angiostatic capacity of sulfated *Escherichia coli* K5 polysaccharide derivatives

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¹The abbreviations used are: BAE cells, bovine aortic endothelial cells; CAM, chorioallantoic membrane; CHO cells, Chinese hamster ovary cells; DMF, *N,N*-dimethylformamide; EGM-2, Endothelial Cell Growth Medium-2; FCS, fetal calf serum; FGF2, basic fibroblast growth factor; FGFR, FGF receptor; GAG, glycosaminoglycan; Glc, glucosamine; GlcA, glucuronic acid; GlcNAc, *N*-acetyl-glucosamine; HGF, hepatocyte growth factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; IdoA, iduronic acid; UFH, unmodified heparin; VEGF, vascular endothelial growth factor.

ABSTRACT

Basic Fibroblast Growth Factor (FGF2) is a potent angiogenic growth factor. FGF2 interacts with tyrosine-kinase receptors (FGFRs) and heparan sulfate proteoglycans (HSPGs) in endothelial cells. Both interactions are required for FGF2-mediated biological responses. Here, we report about the FGF2 antagonist and anti-angiogenic activity of novel sulfated derivatives of the *Escherichia coli* K5 polysaccharide. K5 polysaccharide was chemically sulfated in *N*-position, in *O*-position after *N*-deacetylation, or in both. *O*-sulfated and *N,O*-sulfated K5 derivatives with low and high degree of sulfation compete with immobilized heparin for the binding to ^{125}I -FGF2 with different potency. Accordingly, they abrogate the formation of the HSPG/FGF2/FGFR ternary complex, as evidenced by their capacity to prevent FGF2-mediated cell-cell attachment of FGFR1-overexpressing HSPG-deficient CHO cells to wild-type CHO cells. They also inhibited ^{125}I -FGF2 binding to HSPGs and FGFRs in FGFR1-overexpressing, HSPG-bearing CHO cells and in adult bovine aortic endothelial cells. Also, K5 derivatives inhibited with different potency FGF2-mediated cell proliferation in fetal bovine aortic endothelial GM 7373 cells and in human umbilical vein endothelial (HUVE) cells. In all these assays, the *N*-sulfated K5 derivative and unmodified K5 were poorly effective. Among all the derivatives tested, only highly *O*-sulfated and *N,O*-sulfated K5 derivatives prevented the sprouting of FGF2-transfected murine aortic endothelial FGF2-T-MAE cells in 3D fibrin gel and “spontaneous angiogenesis *in vitro*” on Matrigel of FGF2-T-MAE and HUVE cells. Finally, the highly *N,O*-sulfated K5 derivative exerted a potent anti-angiogenic activity *in vivo* when tested on the chick embryo chorioallantoic membrane. These data demonstrate the possibility to generate FGF2 antagonists endowed with anti-angiogenic activity by specific chemical sulfation of bacterial K5 polysaccharide. In particular, the highly *N,O*-sulfated K5 derivative is effective *in vitro* and *in vivo* on endothelial cells of murine, bovine, avian, and human origin. This compound may provide the basis for the design of novel angiostatic compounds.

INTRODUCTION

Angiogenesis is the process of generating new capillary blood vessels. In the adult, the proliferation rate of endothelial cells is very low compared to many other cell types in the body. Physiological exceptions in which angiogenesis occurs under tight regulation are found in the female reproductive system and during wound healing. Uncontrolled endothelial cell proliferation is observed in tumor neovascularization and in angioproliferative diseases (1). Tumors cannot grow as a mass above few mm³ unless a new blood supply is induced (2). It derives that the control of the neovascularization process may affect tumor growth and may represent a novel approach to tumor therapy (3).

Angiogenesis is controlled by a balance between pro-angiogenic and anti-angiogenic factors (4). Thus, the angiogenic switch represents the net result of the activity of angiogenic stimulators and inhibitors, suggesting that counteracting even a single major angiogenic factor could shift the balance towards inhibition. Major angiogenic factors include, among several others, basic fibroblast growth factor (FGF2)¹ and vascular endothelial growth factor (VEGF). Heparin-binding FGF2 was one of the first to be characterized (5). It induces cell proliferation, chemotaxis, and protease production in cultured endothelial cells (5-7). In vivo, FGF2 shows angiogenic activity in different experimental models (8). In situ hybridization and immunolocalization experiments have shown the presence of FGF2 mRNA and/or protein in neoplastic cells, endothelial cells, and infiltrating cells within human tumors of different origin (9-13). Antisense FGF2 and FGF receptor-1 (FGFR1) cDNAs inhibit neovascularization and growth of human melanomas in nude mice (14). Also, a secreted FGF-binding protein that mobilizes stored extracellular FGF2 can serve as an angiogenic switch for different tumor cell lines, including squamous cell carcinoma and colon cancer cells (15). Interestingly, targeting of FGF-binding protein with specific ribozymes reduces significantly the growth and vascularization of xenografted tumors in mice (16) despite the high levels of VEGF produced by these cells (discussed in ref. 17). These data are in keeping with the synergistic action exerted by the two growth factors in stimulating angiogenesis (18, 19) and with the observation that modulation of FGF2 expression may allow a fine tuning of the angiogenesis process even in the presence of significant levels of VEGF (20). More recently, a recombinant adenovirus expressing soluble FGF receptor has been shown to repress tumor growth and angiogenesis (21). Finally, a significant correlation exists between the presence of FGF2 in cancer cells and advanced tumor stage in human pancreatic (22), renal (23), and colon (24) cancer. Also, interferon- α has been shown to inhibit FGF2 synthesis and to accelerate the regression of vascular tumors (25). Taken together, these data point to FGF2 as a target for anti-angiogenic therapy in tumors.

FGF2 exerts its biological activity on endothelial cells by interacting with high affinity tyrosine-kinase FGFRs (26) and low affinity proteoglycans containing heparan sulfate (HS) as polysaccharide (HSPGs) (27). The physiological effects resulting from the interaction of FGF2 with cell-associated and free HSPGs are manifold. HSPGs protect FGF2 from inactivation in the extracellular environment and modulate the bioavailability of the growth factor (28, 29). At the cell surface, free and cell-associated HSPGs may play contrasting roles in modulating the dimerization of FGF2 and its interaction with FGFRs. For instance, heparin induces FGF2-FGFR interaction in HS-deficient cells (30). This relies on the capacity of the glycosaminoglycan (GAG) to form a ternary complex by interacting with both proteins (31, 32). In apparent contrast with these observations, heparin inhibits the binding of FGF2 to FGFRs when administered to cells bearing surface-associated HSPGs (33). This is probably due to the competition of soluble GAGs with cell-associated HSPGs and FGFRs for the binding to FGF2. Thus, the bioavailability and the biological activity of FGF2 on endothelial cells strictly depend on the extracellular GAG milieu, indicating the possibility of modulating the angiogenic activity of FGF2 *in vivo* by using exogenous GAGs. The capacity of low molecular weight heparin fragments administered systemically to reduce the angiogenic activity of FGF2 supports this hypothesis (34).

A further implementation of this hypothesis is that synthetic molecules and chemically modified heparins able to interfere with the HSPG/FGF2/FGFR interaction may act as angiogenesis inhibitors. In particular, heparin-mimicking, polyanionic compounds able to compete with HSPGs for growth factor interaction may be expected to hamper the binding of FGF2 to the endothelial cell surface with consequent inhibition of its angiogenic capacity. Among such compounds are 6-*O*-desulfated heparin (35), suramin (36), several suramin analogues (37), pentosan polysulfate (38), and sulfonic acid polymers (39).

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 (40) in which GlcA is glucuronic acid and GlcNAc is *N*-acetyl-glucosamine. Previous studies had shown the possibility to generate K5 derivatives by chemical sulfation in *N*- and/or *O*-positions (41). In the present paper we synthesized various *N*-, *O*-, and *N,O*-sulfated K5 derivatives with different degree of sulfation. The compounds were then tested for their FGF2-antagonist activity and anti-angiogenic capacity. The results demonstrate that an highly *N,O*-sulfated K5 derivative binds FGF2 and inhibits the formation of the HSPG/FGF2/FGFR ternary complex, prevents ¹²⁵I-FGF2 binding to endothelial cell surface HSPGs and FGFRs, inhibits FGF2-mediated mitogenesis, sprouting, and morphogenesis in cultured endothelial cells, and blocks angiogenesis in the developing chick embryo chorioallantoic membrane (CAM).

MATERIALS AND METHODS

Materials. Human recombinant FGF2 was from Pharmacia-Upjohn (Milan, Italy). Conventional heparin was obtained from a commercial batch preparation of unfractionated sodium heparin from beef mucosa (1131/900 from Laboratori Derivati Organici S.p.A., Milan, Italy) which was purified from contaminants (purity higher than 95%) according to described methodologies (42). Selectively desulfated heparins were described previously (43) and were a generous gift of Dr. B. Casu, Ronzoni Institute, Milan. Type I heparan sulfate (HS) was from Opocrin (Corlo, Italy). Chondroitin-4-sulfate and dermatan sulfate were a gift of Dr. M. Del Rosso, University of Florence, Italy.

K5 polysaccharide derivatives. All the K5 polysaccharide derivatives (Table 1) were obtained from a single batch of K5 polysaccharide prepared from *E. coli* cell culture grown for 16 h at 37°C in a medium containing 2.0 g/L defatted soy flour, 9.7 g/L K_2HPO_4 , 2.0 g/L KH_2PO_4 , 0.5 g/L sodium citrate, 0.11 g/L $MgCl_2$, 1.0 g/L ammonium sulfate, 2.0 g/L glucose, pH 7.3. Conditioned medium was collected and concentrated 5 times with a 10,000 Da cut-off ultrafiltration membrane. The polysaccharide was recovered after precipitation with 4 volumes of acetone and proteins were hydrolyzed by 90 min incubation at 37°C with protease II from *Aspergillus oryzae* (Sigma, St Louis, MO) in 0.1 M NaCl, 0.15 M EDTA, 0.5% SDS, pH 8.0. K5 polysaccharide was finally recovered by ultrafiltration and precipitation.

A) *O-sulfation of K5 polysaccharide.* One g of K5 polysaccharide was suspended at room temperature in 10 ml of anhydrous *N,N*-dimethylformamide (DMF). Then, 15 ml of DMF containing 2.4 or 0.7 g of pyridine-sulfotrioxide complex were added to generate K5-OS(H) and K5-OS(L), respectively. Samples were incubated for 18 h at room temperature and recovered by precipitation with 16 ml of NaCl-saturated acetone. Pellets were dissolved in water and purified from salts by ultrafiltration.

B) *N-deacetylation/N-sulfation of K5 polysaccharide.* One g of K5 polysaccharide was dissolved in 100 ml of 2.0 N NaOH, incubated for 24 h at 60°C, cooled to room temperature, and the pH was adjusted to 7.0. The solution was warmed up to 40°C, added in a single step with 1.6 g of sodium carbonate and with 1.6 g of pyridine-sulfotrioxide complex stepwise in 4 h, and incubated for further 1 h at the same temperature. The solution was then brought to room temperature and the pH was adjusted to 7.5-8.0. *N*-deacetylated/*N*-sulfated K5 was purified from salts by ultrafiltration and the sample was dried under vacuum.

C) *O*-sulfation of *N*-deacetylated/*N*-sulfated K5 polysaccharide. *N*-deacetylated/*N*-sulfated K5 was dissolved in 10 ml of water, run through a cation exchange column (IR-120 H⁺, Bio Rad) at 10°C, and neutralized with 15% tetrabutylammonium hydroxide in water. After concentration and freeze drying, the sample was dissolved in 40 ml of DMF and added with 0.7 or 3.5 g of pyridine-sulfotrioxide complex in solid form to generate K5-N,OS(L) and K5-N,OS(H), respectively. Samples were incubated at 50°C for 24 h, cooled to 4°C, and added with NaCl-saturated acetone until complete precipitation. After filtration the precipitate was dissolved in 10 ml of water added with NaCl till 0.2 M concentration and the pH was adjusted to 7.5-8.0 with 2.0 N NaOH. The product was recovered by acetone precipitation and ultrafiltration to obtain a 10% solution. Then, samples were warmed up to 40°C, added in a single step with 1.6 g of sodium carbonate and with 1.6 g of pyridine-sulfotrioxide complex stepwise in 4 h, and incubated for further 24 h at the same temperature. Samples were then purified from salts by ultrafiltration.

The ¹³C-NMR spectrum analysis, the sulfate/carboxyl ratio analysis, and the molecular weight determination of the different compounds were performed as described (45-47).

¹²⁵I-FGF2 competition binding to immobilized heparin. Human recombinant FGF2 was labeled with Na¹²⁵I (37 GBq/ml; Amersham International, Amersham, UK) using Iodogen (Pierce Chemical, Rockford, IL) as described (47). Heparin was dissolved at 4.0 mg/ml in 25 mM Tris-HCl, pH 7.5, 130 mM NaCl (TBS). Then, the heparin solution (50 µl/well) was incubated overnight at 37°C in non-tissue culture plastic 96 well-plates as described (48). After washing with TBS, ¹²⁵I-FGF2 was added at 20 ng/ml in the presence of increasing concentrations of the different K5 derivatives. After 2 h of incubation at 4°C, bound radioactivity was collected by 30 min incubation at 50°C in 2% SDS and measured. Nonspecific binding to BSA-coated wells was measured in the absence of any competitor and subtracted from all values.

Cell cultures. Adult bovine aortic endothelial (BAE) cells were provided by M. Pepper (University of Geneva, Switzerland) and grown in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS).

Wild type CHO cells, FGFR1-transfected CHO cells, wild-type CHO-K1 cells [a subclone of CHO cells from where HSPG-deficient mutants were originated (49)], and A745 CHO cell mutants (kindly provided by J.D. Esko, La Jolla, CA) were grown in Ham's F 12 medium supplemented with 10% FCS. A745 CHO cells harbor a mutation which inactivates the xylosyltransferase that catalyzes the first sugar transfer step in GAG synthesis (49). FGFR1-transfected CHO cells and the

A745 CHO *flg-1A* clone, both bearing about 30,000 FGFR1 molecules per cell, were generated in our laboratory by transfection with the IIIc variant of murine FGFR1 cDNA (39).

Balb/c mouse aortic endothelial 22106 cells stably transfected with a human FGF2 cDNA (FGF2-T-MAE cells) (50) were grown in DMEM added with 10% FCS in the presence of 500 µg/ml of G418 sulfate (Sigma, St. Louis, MO).

Transformed fetal bovine aortic endothelial GM 7373 cells, corresponding to the described BFA-1c 1BPT multilayered transformed clone (47) were obtained from the National Institute of General Medical Sciences, Human Genetic Mutual Cell Repository (Camden, NJ). Cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum (FCS), vitamins, and essential and nonessential amino acids.

HUVE cells at passage 3 (Clonetics) were grown in complete Endothelial Cell Growth Medium-2 (EGM-2) (Clonetics).

FGF2-mediated cell-cell adhesion assay. This assay was performed as described (51) with minor modifications. Briefly, wild-type CHO-K1 cells were seeded in 24 well-plates at 52,000 cells/cm². After 24 h, cell monolayers were washed with PBS and incubated with 3% glutaraldehyde in PBS for 2 h at 4°C. Fixation was stopped with 0.1 M glycine and cells were washed extensively with PBS. Then, A745 CHO *flg-1A* cells (52,000 cells/cm²) were added to CHO-K1 monolayers in serum-free medium *plus* 10 mM EDTA with no addition or with 30 ng/ml FGF2 in the absence or in the presence of increasing concentrations of the competitor under test. After 2 h of incubation at 37°C, unattached cells were removed by washing twice with PBS and A745 CHO *flg-1A* cells bound to the wild-type CHO monolayer were counted under an inverted microscope at 125x magnification. Adherent A745 CHO *flg-1A* cells have a rounded morphology and can be easily distinguished from the confluent wild-type CHO monolayer laying underneath on a different plane of focus. Data are expressed as the mean of the cell counts of three microscopic fields chosen at random. All experiments were performed in triplicate and were repeated twice with similar results.

¹²⁵I-FGF2 cell binding assay. Twenty-four hours after plating in 24-well dishes at a density of 70,000 cells/cm², BAE cells or FGFR1-transfected CHO cells were washed three times with ice-cold PBS and incubated for 2 h at 4°C in binding medium (serum-free medium containing 0.15% gelatin, 20 mM Hepes, pH 7.5) with 30 ng/ml of ¹²⁵I-FGF2 in the absence or in the presence of increasing concentrations of the compound under test. Then, after a PBS wash, cells were washed twice with 2 M NaCl in 20 mM Hepes buffer (pH 7.5) to remove ¹²⁵I-FGF2 bound to low affinity

HSPGs and twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0) to remove ^{125}I -FGF2 bound to high affinity FGFRs (47). Nonspecific binding was measured in the presence of 300 $\mu\text{g}/\text{ml}$ suramin and subtracted from all values.

Cell proliferation assays. *Short-term assay:* GM 7373 cells were seeded at 70,000 cells/cm² in 24-well dishes. Plating efficiency was higher than 90%. After overnight incubation, cells were incubated in fresh medium containing 0.4% FCS and 10 ng/ml FGF2. After 8 h, increasing concentrations of the K5 derivative under test were added to cell cultures without changing the medium. Sixteen h thereafter, cells were trypsinized and counted in a Burker chamber. In our experimental conditions, control cultures incubated in 0.4% FCS with no addition or with 10 ng/ml FGF2 undergo 0.1-0.2 and 0.7-0.8 cell population doublings, respectively. Cells grown in 10% FCS undergo 1.0 cell population doublings (52).

Long term assay: HUVE cells were seeded at 2,500 cells/well in 96 well plates in complete EGM-2 medium. After 24 h, all cell cultures were incubated in EGM-2 medium plus 2% FCS without FGF2, VEGF, and heparin. Then wells were added with 30 ng/ml FGF2 in the absence or presence of K5 derivatives. After 3 days cells were stained with crystal violet and plates were read with a microplate reader at 595 nm.

Preparation of three-dimensional gels. FGF2-T-MAE cell aggregates, prepared on agarose-coated plates exactly as described (53), were seeded onto fibrin-coated 48 well-plates. Immediately after seeding, 250 μl of calcium-free medium containing fibrinogen (2.5 mg/ml) and thrombin (250 mU/ml) were added to each well and allowed to gel for 5 min at 37°C. Then, 500 μl of culture medium with or without K5 derivatives (all at 100 $\mu\text{g}/\text{ml}$) were added on the top of the gel. In all the experiments, the fibrinolytic inhibitor trasylol was added to the gel and to the culture medium at 200 KIU/ml to prevent the dissolution of the substrate (53). Formation of radially growing cell sprouts was observed during the next 1-2 days.

Matrigel (Becton Dickinson, Milan, Italy) is an extracellular matrix extract of the murine EHS tumor grown in C57/bl6 mice. 150 $\mu\text{l}/\text{well}$ of Matrigel (10 mg/ml) were used to coat 48 well-plates at 4°C. After gelification at 37°C, HUVE or FGF2-T-MAE cells were seeded onto Matrigel-coated dishes at 40,000 or 75,000 cells/cm², respectively, in the absence or in the presence of K5 derivatives (all at 100 $\mu\text{g}/\text{ml}$). Newly formed endothelial cell “cords” and “tubes” were photographed using an inverted phase contrast photomicroscope.

Chick embryo chorioallantoic membrane (CAM) assay. Fertilized White Leghorn chick eggs were incubated under conditions of constant humidity at 37°C. On the third day of incubation, a square window was opened in the egg shell after removal of 2-3 ml of albumen so as to detach the developing CAM from the shell. The window was sealed with a glass of the same size and the eggs were returned to the incubator. At day 8, 1 mm³ sterilized gelatin sponges (Gelfoam, Upjohn Company, Kalamazoo, USA) adsorbed with the K5-derivative under test (50 µg/embryo) dissolved in 5 µl of PBS were implanted on the top of growing CAMs under sterile conditions (54). Sponges containing vehicle alone were used as negative controls. CAMs were examined daily until day 12 under a Zeiss stereomicroscope SR equipped with the MC 63 Camera System (Zeiss, Oberkochen, Germany) and blood vessels around the sponges were counted. Mean values ± SD were determined for each analysis.

RESULTS

Chemical characterization of K5 derivatives.

Sulfated K5 derivatives were prepared from a single batch of *E. coli* K5 polysaccharide as described in Materials and Methods. One *N*-sulfated derivative (K5-NS), two *O*-sulfated derivatives with low and high degree of sulfation [K5-OS(L) and K5-OS(H), respectively], and two *N,O*-sulfated derivatives with low and high degree of sulfation [K5-N,OS(L) and K5-N,OS(H), respectively] were obtained. Their sulfate/carboxyl ratio (SO₃⁻/COO⁻) values, ¹³C-NMR spectrum analysis, and apparent molecular weight are shown in Table 1. The data demonstrate that the procedure adopted allows a specific sulfation of K5 polysaccharide in *N*- and/or *O*-positions. *N*-deacetylation and *N*-sulfation reactions were brought to completion, resulting in 100% *N*-sulfation of glucosamine (Glc) residues in K5-NS, K5-N,OS(L), and K5-N,OS(H) derivatives. Also, *O*-sulfation resulted in an almost complete 6-*O*-sulfation of Glc residues in all the *O*- and *N,O*-sulfated derivatives that differed for the extent of *O*-sulfation in the other positions. Thus, K5-OS(H) consists of the virtually homogeneous repeat of GlcA2,3SO₃⁻-GlcNAc3,6SO₃⁻ disaccharide units whereas K5-N,OS(H), that shows a similar degree of total sulfation, carries one *N*-sulfated group and one 6-*O*-sulfated group in all Glc residues but is more heterogeneous in the other *O*-positions with 70% of its sequence being represented by GlcA2,3SO₃⁻-GlcNSO₃⁻,6SO₃⁻ disaccharide units (Fig. 1).

Binding of K5 derivatives to ^{125}I -FGF2: competition with immobilized heparin.

K5 derivatives were assessed for the ability to bind ^{125}I -FGF2, thus competing for its interaction with immobilized heparin. To this purpose, ^{125}I -FGF2 was incubated at 4°C in heparin-coated 96 well-plates in the presence of increasing concentrations of the various derivatives. After 2 h, the amount of radioactivity bound to the substrate was measured. Preliminary experiments had shown that ^{125}I -FGF2 interaction with immobilized heparin is specific and is competed by free heparin with an ID_{50} equal to approximately 10 ng/ml (data not shown). As shown in Fig. 2, K5-OS(H) and K5-N,OS(H) derivatives compete for the binding of ^{125}I -FGF2 to immobilized heparin with a potency similar to that shown by heparin ($\text{ID}_{50} = 10$ ng/ml) whereas K5-N,OS(L) is approximately 30 times less effective. In contrast, unmodified K5 and the derivatives K5-NS and K5-OS(L) do not exert a significant competition ($\text{ID}_{50} > 3.0$ $\mu\text{g/ml}$).

Effect of K5 derivatives on HSPG/FGF2/FGFR ternary complex-mediated cell-cell adhesion.

FGF2 has been demonstrated to mediate cell attachment by linking FGFRs and HSPGs on neighboring cells via the formation of HSPG/FGF2/FGFR ternary complexes (51). Heparin/HS-like compounds endowed with the capacity to bind FGF2 can be anticipated to hamper the formation of the HSPG/FGF2/FGFR ternary complex and to inhibit FGF2-mediated cell-cell interaction (39). To assess the impact of K5 derivatives on the formation of the HSPG/FGF-2/FGFR ternary complex, we utilized an experimental model in which the disruption of the complex abolishes FGF2-mediated cell-cell attachment of HSPG-deficient CHO mutants transfected with FGFR1 to wild-type CHO-K1 cells expressing HSPGs but not FGFR (51). To this purpose, preliminary experiments were performed to characterize this model.

As shown in Fig. 3A, FGF2 allows the adhesion of FGFR1-transfectants to the HSPG-bearing cell monolayer in a dose dependent manner ($\text{ED}_{50} = 10$ ng/ml). Only a very limited number of FGFR-1-transfectants adhere instead to the monolayer in the absence of the growth factor (20 ± 4 cells per field). Specificity of the interaction is demonstrated by the lack of activity of the heparin-binding growth factors VEGF₁₆₅ and hepatocyte growth factor (HGF), and of the basic protein cytochrome c (Fig. 3A). Also, FGF2-mediated cell-cell adhesion was not observed when one of the two cell types lacked HSPGs or FGFRs or when both cell types expressed HSPGs, FGFRs, or none of the two receptors (Fig. 3B). Thus, these data demonstrate that cell-cell adhesion in this model is specific for FGF2 and depends on the expression of HSPG and FGFR1 on neighboring cells.

Various GAGs and selectively desulfated heparins differently affect the formation of the HSPG/FGF2/FGFR ternary complex in this model. Indeed, heparin is approximately 10 times more potent than HS in preventing FGF2-mediated cell-cell attachment whereas dermatan sulfate and chondroitin-4-sulfate are ineffective (Fig. 3C). Also, selective *N*-desulfation/*N*-acetylation completely abolished the antagonist activity of heparin, whereas selective 2-*O*-desulfation or 6-*O*-desulfation caused a 30-100-fold decrease in its potency (Fig. 3D).

On this basis, the capacity of sulfated K5 derivatives to affect FGF2-mediated cell-cell attachment was investigated. As shown in Fig. 4A, K5 and *N*-sulfated K5 (K5-NS) were ineffective at doses up to 100 $\mu\text{g/ml}$. In contrast, *O*-sulfation was able to confer to K5 derivatives an inhibitory activity related to their degree of *O*-sulfation. Indeed, the highly *O*-sulfated K5 derivative K5-OS(H) ($\text{SO}_3^-/\text{COO}^- = 3.77$) exerts an antagonist activity ($\text{ID}_{50} = 0.1 \mu\text{g/ml}$) 100 times more potent than that of low *O*-sulfated K5-OS(L) ($\text{SO}_3^-/\text{COO}^- = 1.41$; $\text{ID}_{50} = 10 \mu\text{g/ml}$) and similar to that of unmodified heparin ($\text{SO}_3^-/\text{COO}^- = 2.14$). Interestingly, K5-N,OS(L) and K5-N,OS(H) derivatives, that carry both *N*- and *O*-sulfated groups and show a different degree of sulfation ($\text{SO}_3^-/\text{COO}^-$ equal to 1.70 and 3.84, respectively), exerted an inhibitory activity 10 times more potent ($\text{ID}_{50} = 0.01 \mu\text{g/ml}$) than that of K5-OS(H) and unmodified heparin.

When K5 derivatives were assessed for the capacity to inhibit the binding of ^{125}I -FGF2 to wild type CHO cells transfected with FGFR1 cDNA (that is to CHO cells bearing both HSPGs and FGFRs on their cell surface), the results shown in Fig. 4B were obtained. At the dose of 10 $\mu\text{g/ml}$, K5-OS(H), K5-N,OS(L), and K5-N,OS(H) were able to prevent the binding of ^{125}I -FGF2 to both HSPGs and FGFRs whereas K5-OS(L) prevented FGFR1 interaction only. No inhibitory effect was instead exerted by K5 and K5-NS. When the antagonist activity of K5-N,OS(H) was assessed in a dose-response experiment, the results demonstrated that the compound prevented the binding of ^{125}I -FGF2 to HSPGs and FGFR1 with an ID_{50} equal approximately to 0.3-1.0 $\mu\text{g/ml}$, that is with a potency 100 times lower than that required to prevent the formation of the HSPG/FGF2/FGFR1 ternary complex in the cell-cell adhesion assay (Fig. 4C).

Effect of K5 derivatives on cell-surface binding capacity and biological activity exerted by FGF2 on cultured endothelial cells.

Next, we assessed the ability of K5 derivatives to prevent the binding of ^{125}I -FGF2 to low affinity HSPGs and high affinity tyrosine kinase FGFRs in endothelial cells. As shown in Fig. 5, K5 derivatives inhibit ^{125}I -FGF2 binding to low and high affinity sites in BAE cells with different potency. As observed for CHO cells, K5-OS(H), K5-N,OS(L), and K5-N,OS(H) caused inhibition

of ^{125}I -FGF2 binding to HSPGs and FGFRs with similar efficacy. K5-OS(L) appeared to exert an intermediate effect whereas unmodified K5 and K5-NS were ineffective.

FGF2 is able to induce an angiogenic phenotype in endothelial cells either when added exogenously to cell cultures or when produced endogenously. This phenotype includes, among other responses, endothelial cell proliferation, sprouting, and morphogenesis (53, 55, 56). To evaluate a possible angiosuppressive activity of K5 derivatives, we evaluated their capacity to affect FGF2-mediated cell proliferation in bovine aortic endothelial GM 7373 cells and in HUVE cells in short-term and long-term proliferation assays, respectively. In both assays, K5-N,OS(H) inhibits endothelial cell proliferation in a dose-dependent manner (ID_{50} equal to approximately 10-30 $\mu\text{g/ml}$) whereas unmodified K5 was ineffective (Fig. 6A). When all the derivatives were tested at the dose of 100 $\mu\text{g/ml}$, only K5-N,OS(H) appeared to exert a significant inhibitory activity on HUVE cells whereas K5-OS(L), K5-OS(H), K5-N,OS(L), and K5-N,OS(H) were active on FGF2-mediated cell proliferation in GM 7373 cells (Fig. 6B), possibly reflecting structural differences in cell surface HSPG composition of the two cell types.

The capacity of K5 derivatives to affect the first steps of the angiogenesis process was investigated further by the *in vitro* sprout formation assay (53). In this assay, FGF2-transfected murine aortic endothelial FGF2-T-MAE cell aggregates are embedded into the fibrin gel and the formation of radially growing endothelial sprouts follows (53). Accordingly, FGF2-T-MAE cell aggregates invade the gel and form solid sprouts after 1-2 days in culture in the absence of any competitor (Fig. 7). One hundred $\mu\text{g/ml}$ of K5-OS(H) or of K5-N,OS(H) fully prevented sprout formation whereas unmodified K5 and all the other derivatives were ineffective.

The late phase of neovascularization is characterized by the evolution of solid endothelial cell sprouts into capillary tubes. *In vitro*, the culture of different endothelial cell types on Matrigel, a laminin-rich gelled basement membrane matrix, results in the formation of vascular tubes, a phenomenon known as “spontaneous angiogenesis *in vitro*” (53, 57). Thus, to evaluate whether K5 derivatives can affect also the late, differentiative steps of the angiogenesis process, all the compounds were tested for the capacity to prevent vascular tube formation on Matrigel by FGF2-T-MAE and HUVE cells (Fig. 7). Again, only K5-OS(H) and K5-N,OS(H) exerted a significant inhibitory effect on the morphogenesis of the two endothelial cell types when tested at 100 $\mu\text{g/ml}$.

Effect of K5 derivatives on angiogenesis in the chick embryo CAM.

Previous observations from our laboratory had shown that the chick embryo CAM expresses FGF2 mRNA and protein (58). Endogenous FGF2 plays a limiting role in the development of the

vascular system of this embryonic membrane (7). On this basis, the CAM represents an *in vivo* system suitable to assess the impact of putative anti-angiogenic FGF2 antagonists on blood vessel formation. As shown in Fig. 8, K5-N,OS(H) exerts a potent inhibitory activity on blood vessel formation when applied on the top of the CAM at 50 $\mu\text{g}/\text{embryo}$. This results in a rapid and significant decrease in the number of blood vessels surrounding the implant. In contrast, all the other K5 derivatives tested were ineffective.

DISCUSSION

A dual receptor mechanism has been proposed for FGF2 in which interaction of the growth factor with non-signaling low affinity HSPGs is required for the binding to the high affinity tyrosine kinase FGFRs. FGFR occupancy will then trigger an intracellular signal cascade leading to multiple biological responses, including cell proliferation, migration, differentiation, protease production, and angiogenesis (59).

The capsular K5 polysaccharide from *Escherichia coli* has the same structure $[\rightarrow 4)\text{-}\beta\text{-D-GlcA-(1}\rightarrow 4)\text{-}\alpha\text{-D-GlcNAc-1(1}\rightarrow]_n$ as the heparin precursor *N*-acetyl heparosan (40). In the present paper we synthesized *N*-, *O*-, and *N,O*-sulfated K5 derivatives with different degree of sulfation. The procedure here adopted allows a specific sulfation of K5 polysaccharide in *N*- and/or *O*-positions. Indeed, *N*-deacetylation/*N*-sulfation reactions resulted in the complete *N*-sulfation of Glc residues whereas *O*-sulfation resulted in the almost complete 6-*O*-sulfation of Glc residues in all the *O*- and *N,O*-sulfated derivatives that differed therefore for the extent of *O*-sulfation in the other positions (see Table 1).

Our data indicate that K5 derivatives with specific chemical sulfation may act as FGF2 antagonists by mimicking some of the functional features of heparin/HS. Indeed, sulfated K5 derivatives interact with FGF2 by competing with immobilized heparin for the binding to ^{125}I -FGF2. X-ray crystallography has identified a cluster of noncontiguous positively-charged amino acids in the 3D structure of FGF2 that form a "basic region" able to interact with sulfate groups of heparin (60, 61). Our results indicate that sulfate groups of K5 derivatives mimic sulfated heparin in its interaction with FGF2. To this respect, it must be pointed out that nonsulfated K5 polysaccharide is devoid of any significant FGF2-binding capacity, thus indicating that sulfate groups are essential for the interaction.

Heparin consists largely of 2-*O*-sulfate IdoA \rightarrow *N*,6-*O*-disulfate Glc disaccharide units. Other disaccharides containing unsulfated IdoA or GlcA and *N*-sulfated or *N*-acetylated Glc are also present as minor components. This heterogeneity is more pronounced in HS, where the low-sulfated

disaccharides are the most abundant. The minimal FGF2-binding sequence in HS has been identified as a pentasaccharide that contains the disaccharide units IdoA2SO₃⁻-GlcNSO₃⁻ or IdoA2SO₃⁻-GlcNSO₃⁻,6SO₃⁻ (62). Accordingly, studies using selectively desulfated heparins revealed an absolute requirement for *N*- and 2-*O*-sulfate groups in FGF2 binding, whereas 6-*O*-sulfates may be involved in FGFR binding and formation of the HSPG/FGF2/FGFR ternary complex (32).

Our data indicate that highly sulfated K5-OS(H), that consists of the virtually homogeneous repeat of GlcA2,3SO₃⁻-GlcNAc3,6SO₃⁻ disaccharide units, and K5-N,OS(H), 70% of its sequence being represented by GlcA2,3SO₃⁻-GlcNSO₃⁻,6SO₃⁻ disaccharide units, interact with FGF2 with an affinity similar to that shown by heparin. K5-N,OS(L) shows an intermediate potency, whereas K5-NS and K5-OS(L) are ineffective. This suggests that both the degree of sulfation and charge distribution modulate the FGF2-binding capacity of sulfated K5 derivatives that require sulfation of GlcA residues, but not epimerization, for an high affinity interaction with the growth factor.

K5 derivatives abrogate FGF2-mediated attachment of HSPG-deficient FGFR1-transfected CHO mutants to a monolayer of wild-type HSPG-bearing CHO-K1 cells, thus indicating their ability to prevent the formation of the HSPG/FGF2/FGFR ternary complex. Likewise, they impair the binding of ¹²⁵I-FGF2 to both HSPGs and FGFRs in cultured endothelial cells. The different capacity of the various K5 derivatives to prevent FGF2-mediated cell-cell interaction appears to reflect, at least in part, their different ability to inhibit the binding of FGF2 to HSPGs and/or FGFRs. Indeed, K5-OS(H), K5-N,OS(L), and K5-N,OS(H) were able to prevent the binding of ¹²⁵I-FGF2 to both HSPGs and FGFRs in FGFR1-transfected CHO cells whereas K5-OS(L) prevented FGFR1 interaction only. Interestingly, K5-N,OS(H) prevented the binding of ¹²⁵I-FGF2 to HSPGs and FGFR1 with a potency 100 times lower than that required to prevent the formation of the HSPG/FGF2/FGFR1 ternary complex in the cell-cell adhesion assay. This suggests that the capacity of K5 derivatives to affect the formation of the ternary complex is the synergistic consequence of their inhibitory action on the binding of FGF2 to low and high affinity receptors.

Synthetic molecules and chemically modified heparins able to interfere with HSPG/FGF2/FGFR interaction may act as angiogenesis inhibitors. In particular, heparin-mimicking, polyanionic compounds that are able to compete with HSPGs for growth factor interaction may be expected to hamper the binding of FGF2 to the endothelial cell surface with consequent inhibition of its angiogenic capacity (35-39). Our data indicate that sulfated K5 derivatives can affect cell interaction and biological activity of FGF2 in endothelial cells. For each compound, the antagonist activity varies according to the endothelial cell type and/or the biological assay utilized for the screening. For instance, only K5-N,OS(H) exerts a significant inhibitory

activity on FGF2-mediated HUVE cell proliferation whereas K5-OS(L), K5-OS(H), K5-N,OS(L), and K5-N,OS(H) were all active on bovine aortic GM 7373 cells, possibly reflecting structural differences in cell surface HSPG composition of the two endothelial cell types. Nevertheless, among the derivatives tested, only K5-N,OS(H) exerted a significant antagonist activity in all the *in vitro* biological assays aimed to mimic the early and late phases of the angiogenesis process, including FGF2-mediated mitogenesis, sprouting, and morphogenesis in cultured endothelial cells of bovine, murine, and human origin. Also, K5-N,OS(H) was the only K5 derivative able to prevent the vascularization of the chick embryo CAM.

These data indicate that both the degree of sulfation and charge distribution modulate the biological activity of sulfated K5 derivatives. Indeed, highly sulfated K5-OS(H) and K5-N,OS(H) (with a $\text{SO}_3^-/\text{COO}^-$ equal approximately to 3.8 for both compounds) are equally effective on endothelial cells in most of the *in vitro* assays whereas their low sulfated counterparts K5-OS(L) and K5-N,OS(L) are poorly effective. As pointed out above, K5-OS(H) consists of the virtually homogeneous repeat of $\text{GlcA}_{2,3}\text{SO}_3^-$ - $\text{GlcNAc}_{3,6}\text{SO}_3^-$ disaccharide units whereas 70% of K5-N,OS(H) sequence is represented by $\text{GlcA}_{2,3}\text{SO}_3^-$ - GlcNSO_3^- , $_{6}\text{SO}_3^-$ disaccharide units. This suggests that the degree of sulfation of K5 derivatives, rather than their charge distribution, may be a major determinant for their FGF2-antagonist activity in our *in vitro* assays. In contrast, *N*-sulfation confers an unique *in vivo* angiostatic activity to K5-N,OS(H) that is not observed with K5-OS(H). Thus, *N*-sulfation represents an absolute requirement for the angiostatic activity of K5 derivatives that must be sulfated also in *O* position(s). To this respect, the lack of angiostatic activity of K5-N,OS(L) indicates that the almost complete *N*-sulfation and 6-*O*-sulfation of the Glc residues is not sufficient to confer an angiostatic capacity to K5. Thus, sulfation in 2-*O*- and/or 3-*O*-position in GlcA residues and/or in 3-*O*-position in Glc residues are also required. Indeed, preliminary observations have shown that a limited (40-50%) sulfation in 2-*O*- and 3-*O*-positions in GlcA residues is sufficient to confer to K5-N,OS a significant angiostatic activity *in vivo* (M. Presta and D. Ribatti, data not shown). In agreement with our observations, previous findings had shown a limited capacity of non-sulfated K5 to affect angiogenesis in the CAM assay when compared to HS-derived oligosaccharides (63). The identification of the minimal sulfation pattern required for a full angiostatic activity in K5 derivatives deserves further investigation.

It must be pointed out that angiogenesis occurs in the CAM assay in the absence of exogenously added angiogenic stimuli, indicating that K5-N,OS(H) acts on endogenously released angiogenic factors. Among them, endogenous FGF2 is a likely candidate. Indeed, neutralizing anti-FGF2 antibodies decrease significantly the angiogenic process during CAM development (7). However, various angiogenic factors besides FGF2 (including other members of the FGF family,

isoforms of VEGF and placenta growth factor, HGF, angiogenin, interleukin-8, and HIV-1 Tat protein) share the capacity to interact with heparin and may thus be assumed to interact with sulfated K5 derivatives as well. Further studies are required to assess the susceptibility of the various heparin-binding angiogenic factors to this class of compounds.

In conclusion, the highly *N,O*-sulfated K5 derivative exerts a potent FGF2 antagonist and angiostatic activity, being effective *in vitro* and *in vivo* on endothelial cells of murine, bovine, avian, and human origin. K5-N,OS(H) is endowed with a low anti-coagulant activity when compared to heparin (approximately 20%; P. Oreste and G. Zoppetti, unpublished observations). K5-N,OS(H) may therefore provide the basis for the design of novel angiostatic compounds with therapeutic implications in different angiogenesis-dependent diseases.

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Table 1. Chemical characterization of K5 derivatives.

| sample | SO ₃ ⁻ /COO ⁻ ratio | Glc-NSO ₃ ⁻ (%) | Glc-6SO ₃ ⁻ (%) | *GlcA-OSO ₃ ⁻ (%) | GlcA2,3SO ₃ ⁻ (%) | non-sulfated GlcA (%) | molecular weight (Da) |
|------------|---|--|--|--|--|--------------------------|--------------------------|
| K5 | 0 | 0 | 0 | 0 | 0 | 100 | 30,000 |
| K5-NS | 1.00 | 100 | 0 | 0 | 0 | 100 | 15,000 |
| K5-OS(L) | 1.41 | 0 | 90 | <10 | 0 | > 90 | 14,000 |
| K5-OS(H) | 3.77 | 0 | 100 | 0 | 100 | 0 | 11,000 |
| K5-N,OS(L) | 1.70 | 100 | 90 | <10 | 0 | > 90 | 13,000 |
| K5-N,OS(H) | 3.84 | 100 | 100 | 30 | 70 | 0 | 15,000 |

¹³C-NMR spectrum analysis, sulfate/carboxyl ratio (SO₃⁻/COO⁻) analysis, and molecular weight determination were performed as described in Materials and Methods.

* GlcA2SO₃⁻ or GlcA3SO₃⁻.

LEGEND TO THE FIGURES

Figure 1. Predominant K5 derivative components. The most frequent disaccharide unit present in each K5 derivative (ranging from 70% to 100% of total) is shown as inferred by $\text{SO}_3^-/\text{COO}^-$ and ^{13}C -NMR analysis (see Table 1).

Figure 2. Effect of K5 derivatives on ^{125}I -FGF2 binding to immobilized heparin. ^{125}I -FGF2 (20 ng/ml) was incubated at 4°C in heparin-coated 96 well-plates in the presence of increasing concentrations of K5 (○), K5-NS (□), K5-OS(L) (△), K5-OS(H) (▲), K5-N,OS(L) (◇) or K5-N,OS(H) (◆). After 2 h, the amount of radioactivity bound to the substrate was measured. Nonspecific binding to BSA-coated wells was measured in the absence of any competitor and subtracted from all values.

Figure 3. FGF2-mediated cell-cell attachment. **A)** HSPG-deficient FGFR1 transfectants were added to wild type CHO monolayers in serum-free medium with increasing concentrations of FGF2 (●), or with 100 ng/ml VEGF (○), HGF (■), or cytochrome c (□). **B)** Different CHO cell types expressing or not HSPGs or FGFR1 were suspended in serum-free medium *plus* 30 ng/ml FGF2 and added to different CHO monolayers in various combinations. FR1: HSPG-deficient FGFR1-transfected CHO cells expressing FGFR1 only; HSPG: wild type CHO cells expressing HSPGs only; no: HSPG-deficient CHO cells expressing neither FGFR1 nor HSPGs. **C)** HSPG-deficient FGFR1 transfectants were added to wild type CHO monolayers in serum-free medium with 30 ng/ml FGF2 in the presence of increasing concentrations of heparan sulfate (HS, ○), chondroitin-4-sulfate (Ch-4-S, □), dermatan sulfate (DS, ▲), or unmodified heparin (UFH, ●). **D)** HSPG-deficient FGFR1 transfectants were added to wild type CHO monolayers in serum-free medium with 30 ng/ml FGF2 in the presence of increasing concentrations of 2-*O*-desulfated (○), 6-*O*-desulfated (□), *N*-desulfated/*N*-acetylated (■), or unmodified (●) heparin. For all the experiments, cells bound to the monolayer were counted under an inverted microscope after 2 hr of incubation at 37°C. The data are the mean \pm SD of experiments performed in triplicate that were repeated twice with similar results.

Figure 4. Effect of K5 derivatives on FGF2-mediated cell-cell adhesion. **A)** HSPG-deficient FGFR1 transfectants were added to wild type CHO monolayers in serum-free medium with 30 ng/ml FGF2 in the presence of increasing concentrations of K5 (○), K5-NS (□), K5-OS(L) (△), K5-OS(H) (▲), K5-N,OS(L) (◇) or K5-N,OS(H) (◆). After 2 hr of incubation at 37°C, the bound cells were counted under an inverted microscope. Experiments were performed in triplicate and were repeated twice with similar results. **B)** FGFR1-transfected CHO cells were incubated with 30 ng/ml ¹²⁵I-FGF2 in the absence or in the presence of 10 μg/ml of the different K5 derivatives. After 2 h of incubation at 4°C, the radioactivity associated with HSPGs (black bars) and FGFRs (open bars) was measured. Briefly, cells were washed twice with 2 M NaCl in 20 mM Hepes buffer (pH 7.5) to collect ¹²⁵I-FGF2 bound to HSPGs and then twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0) to collect ¹²⁵I-FGF2 bound to FGFRs. Nonspecific binding was measured in the presence of 300 μg/ml suramin and subtracted from all values. Data are expressed as percent of the binding in the absence of the compound. Similar results were obtained in three independent experiments. **C)** FGFR1-transfected CHO cells were incubated with 30 ng/ml ¹²⁵I-FGF2 in the presence of increasing concentrations of K5-N,OS(H). After 2 h of incubation at 4°C, the radioactivity associated with HSPGs (▲) and FGFRs (△) was measured as above and compared to the capacity of the compound to affect FGF2-mediated cell-cell attachment (●) as described in the legend to Figure 2A. *, statistically different from control (p < 0.05 or better, Student's *t* test).

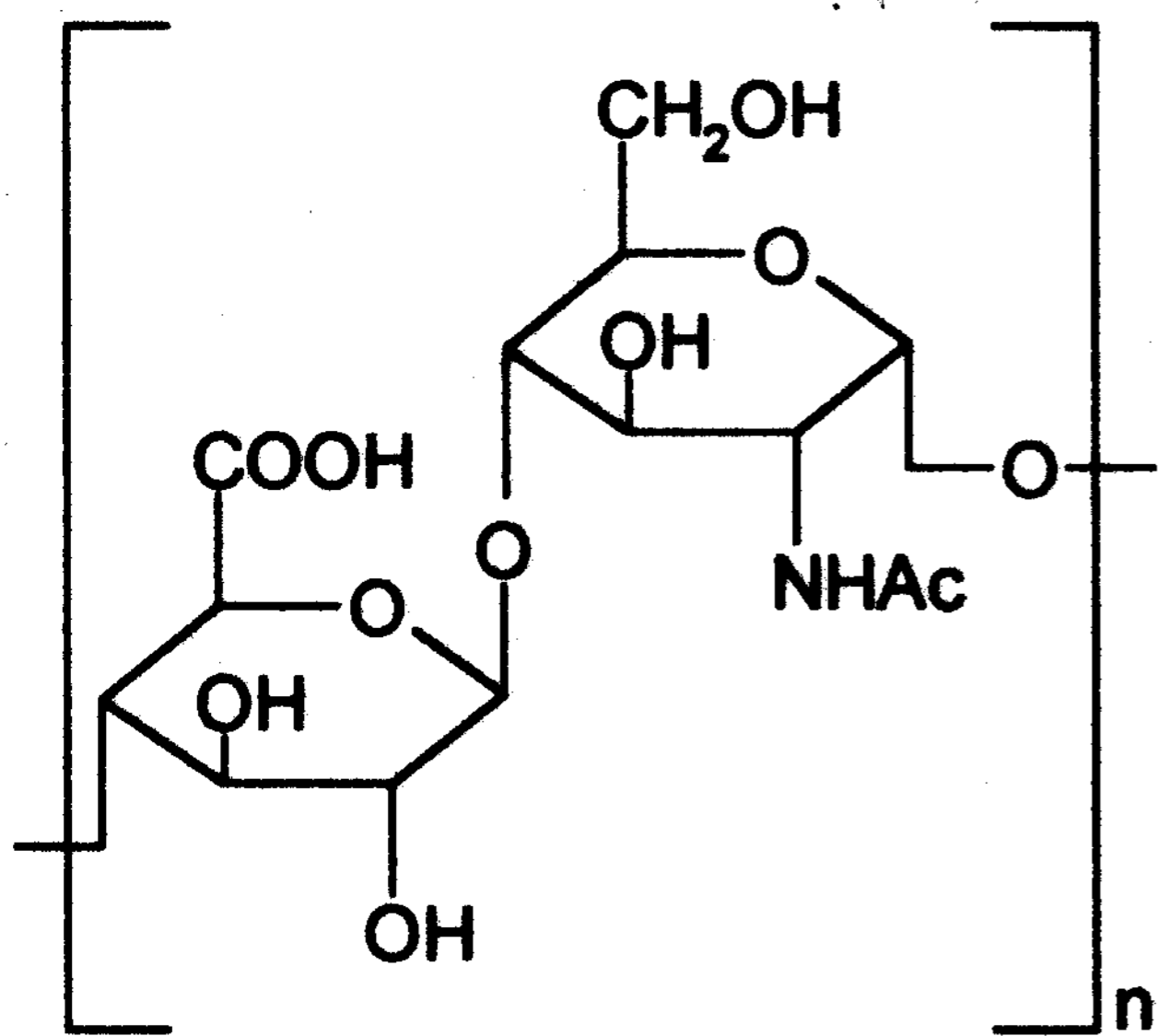
Figure 5. Effect of K5 derivatives on the binding of ¹²⁵I-FGF2 to endothelial cells. BAE cell cultures were incubated with 30 ng/ml ¹²⁵I-FGF2 in the absence or in the presence of 10 μg/ml of the different K5 derivatives. After 2 h of incubation at 4°C, the radioactivity associated with HSPGs (black bars) and FGFRs (open bars) was measured. Data are expressed as percent of the binding in the absence of the compound. *, statistically different from control (p < 0.05 or better, Student's *t* test).

Figure 6. Effect of K5 derivatives on the mitogenic activity of FGF2 in endothelial cells. **A)** Increasing concentrations of unmodified K5 (▲, △) and of K5-N,OS(H) (●, ○) were tested for the capacity to inhibit FGF2-mediated proliferation in a short-term assay on GM 7373 cells (open symbols) and in a long-term assay on HUVE cells (closed symbols) as described in Materials and Methods. **B)** K5 derivatives (all at 100 μg/ml) were tested as in panel A on GM 7373 cells (open bars) and HUVE cells (black bars). Data are expressed as percent of the proliferation measured in

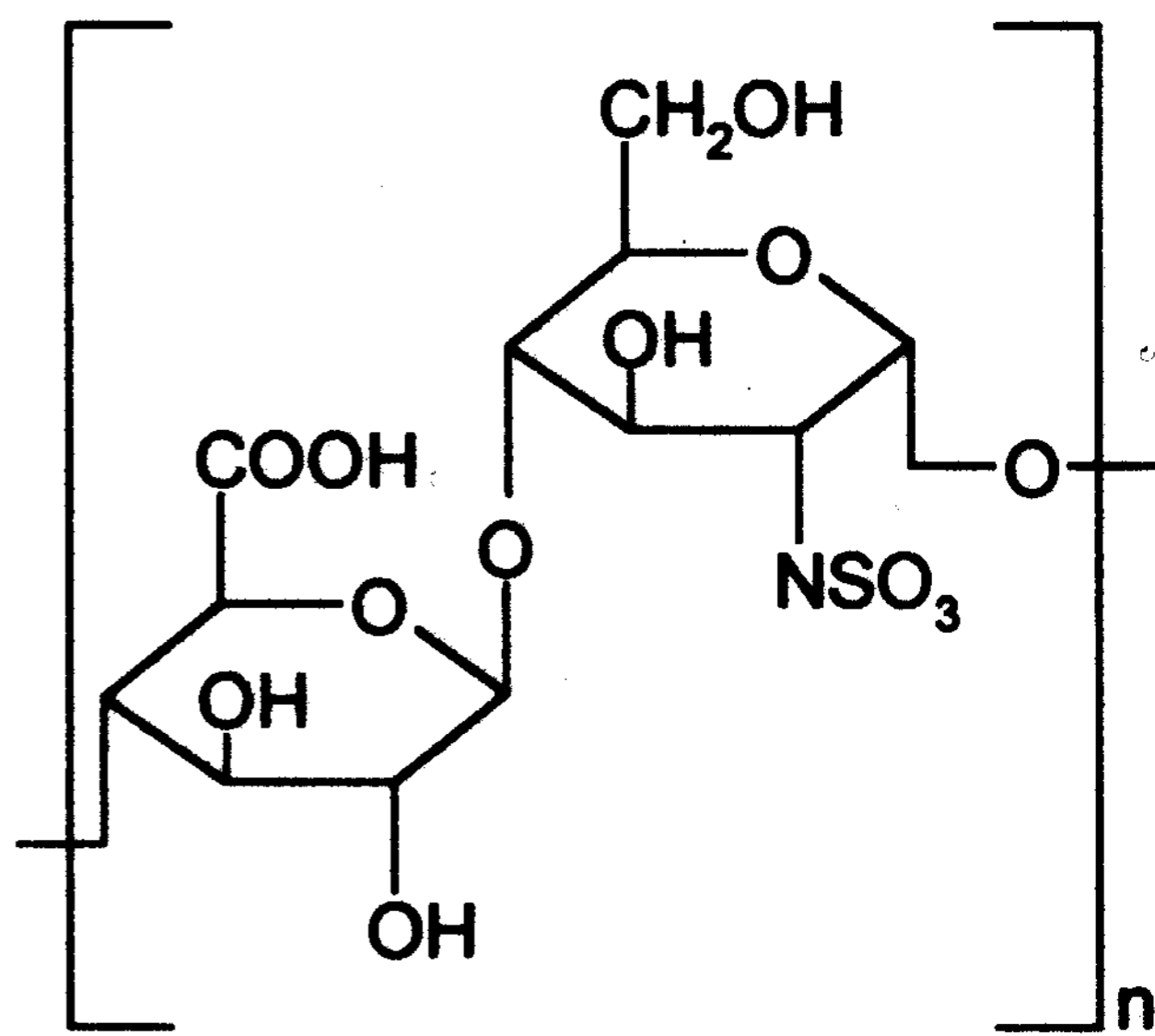
the absence of any competitor. *, statistically different from control ($p < 0.05$ or better, Student's t test).

Figure 7. Effect of K5 derivatives on in vitro endothelial cell sprouting and morphogenesis. FGF2-T-MAE cells (*a-f*) and HUVE cells (*g-i*) were seeded in 3D-fibrin gel (*a-c*) or Matrigel (*d-i*) with no addition or in the presence of K5-OS(H) or K5-N,OS(H) (both at 100 $\mu\text{g/ml}$). After 48 h, cells were photographed under an inverted microscope. Note the inhibition of endothelial cell sprouting and morphogenesis in cell cultures treated with K5-OS(H) or K5-N,OS(H). No inhibition was observed in cell cultures treated with K5, K5-NS, K5-OS(L), or K5-N,OS(L) (not shown). Original magnification: 40x.

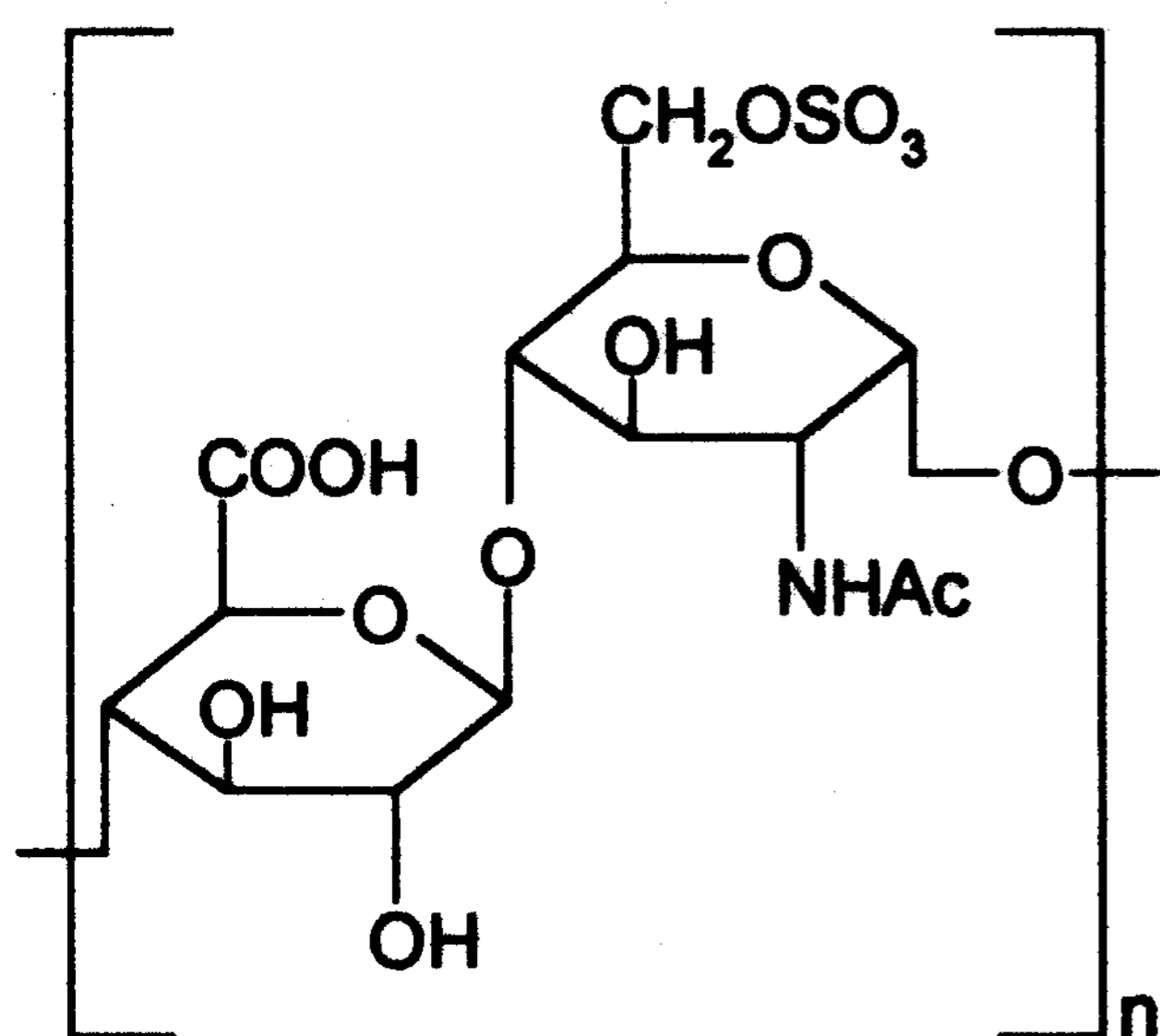
Figure 8. Effect of K5 derivatives on chick embryo CAM vascularization. Gelatin sponges adsorbed with of K5 (○), K5-NS (□), K5-OS(L) (△), K5-OS(H) (▲), K5-N,OS(L) (◇) or K5-N,OS(H) (◆) (all at 50 $\mu\text{g/embryo}$) were implanted on the top of growing CAMs at day 8. Sponges containing vehicle alone were used as negative controls. CAMs were examined daily and blood vessels around the sponges were counted until day 12. Each point is the mean \pm SD of 4 embryos.



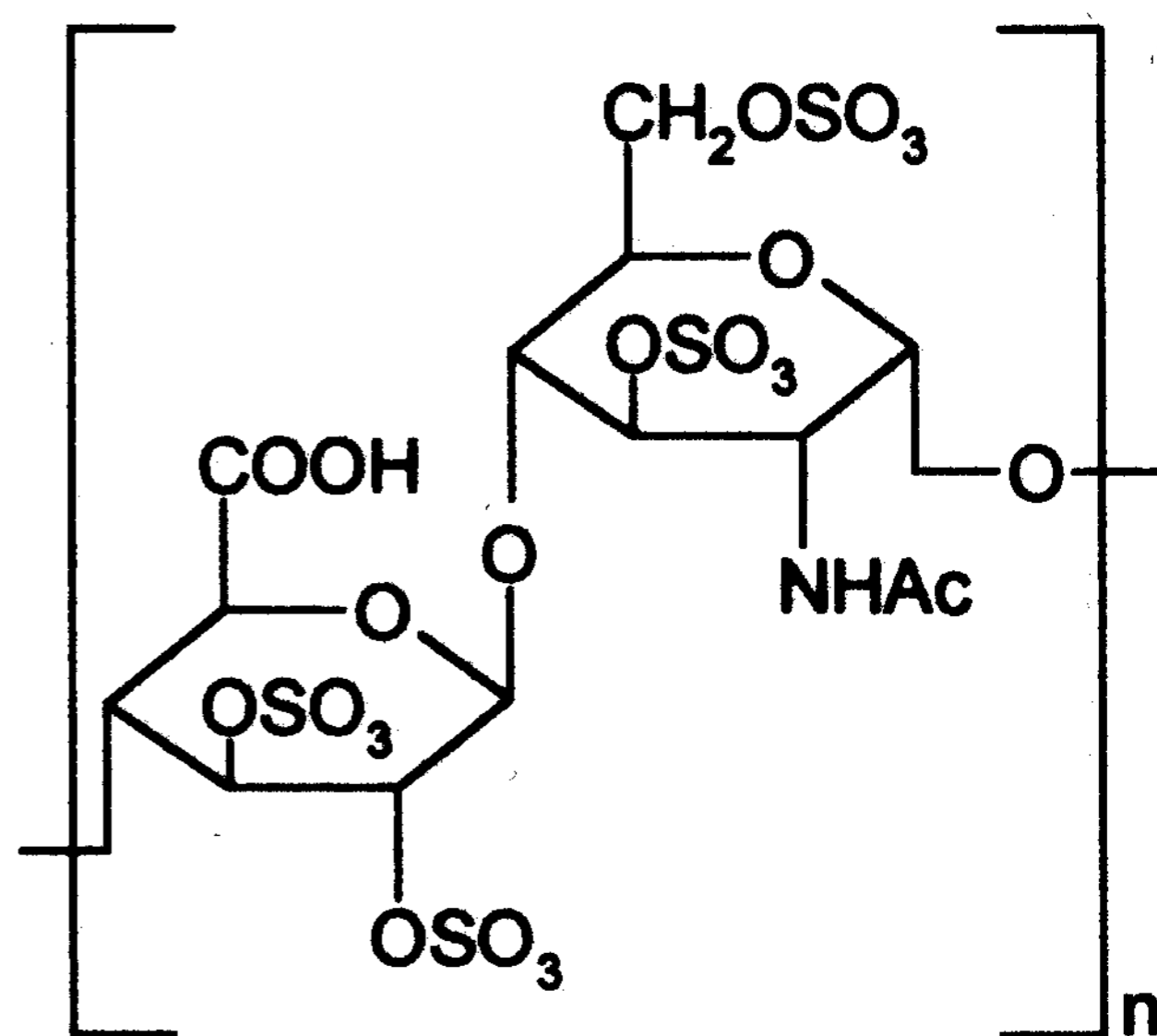
K5



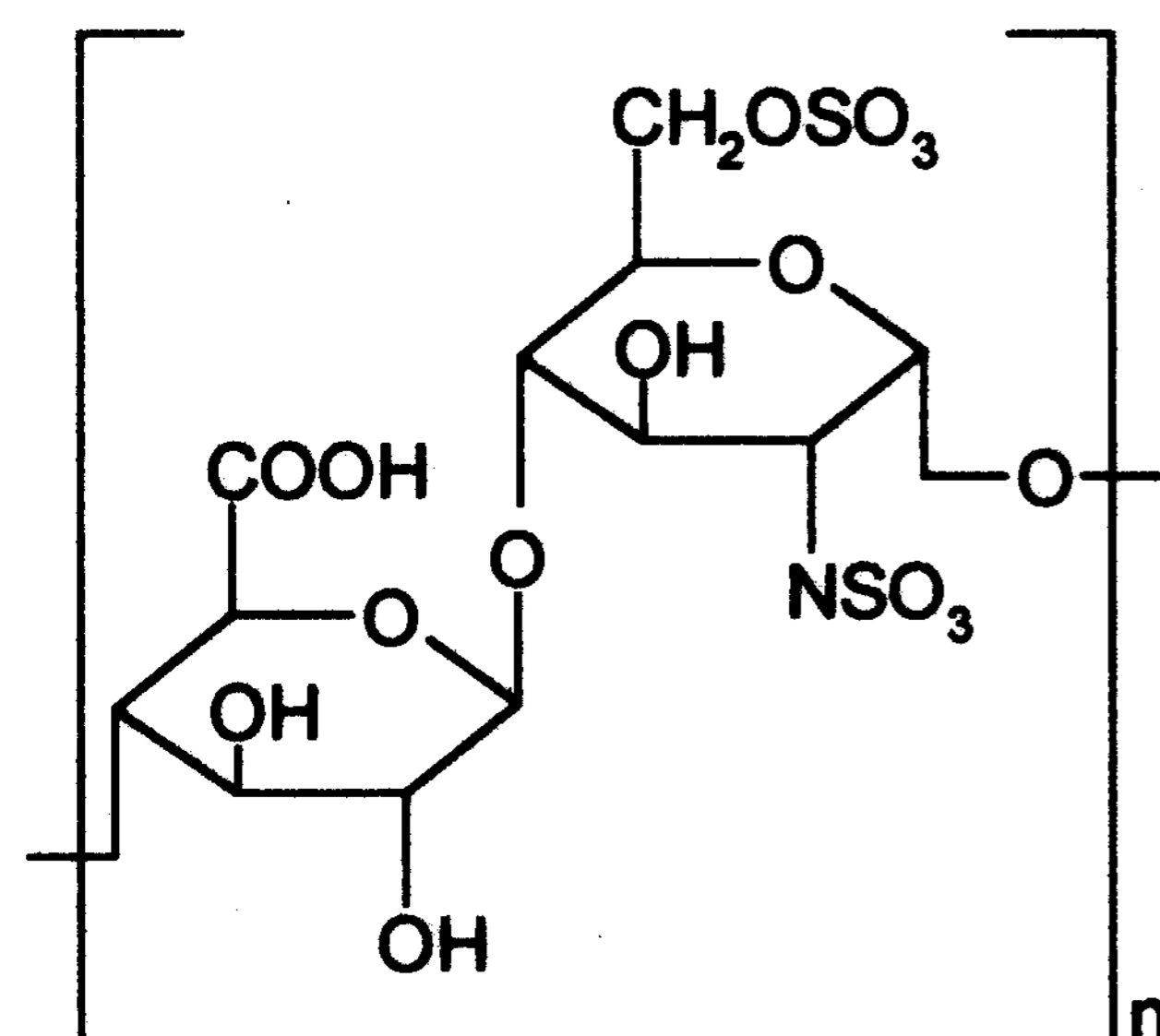
K5-NS



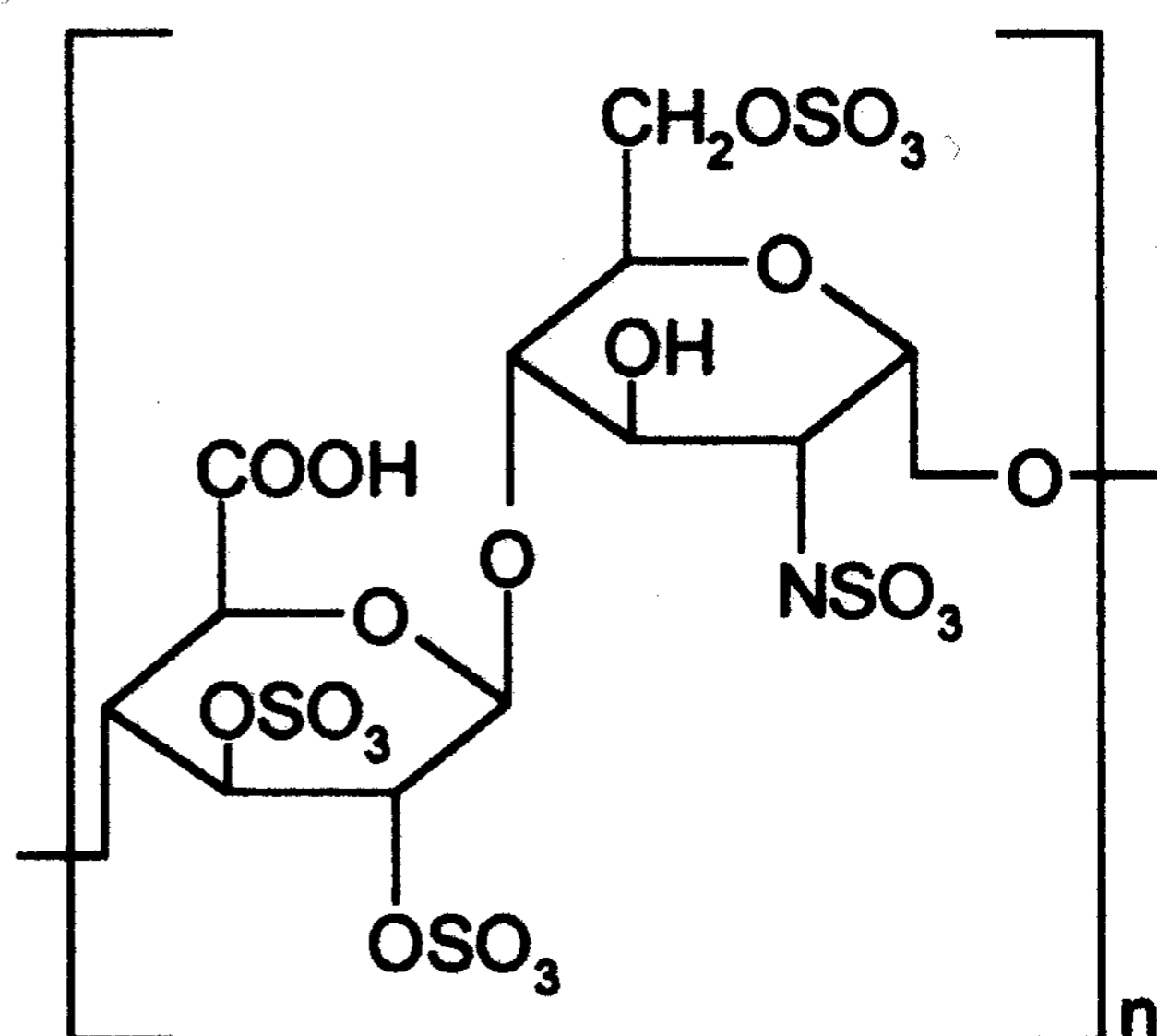
K5-OS(L)



K5-OS(H)

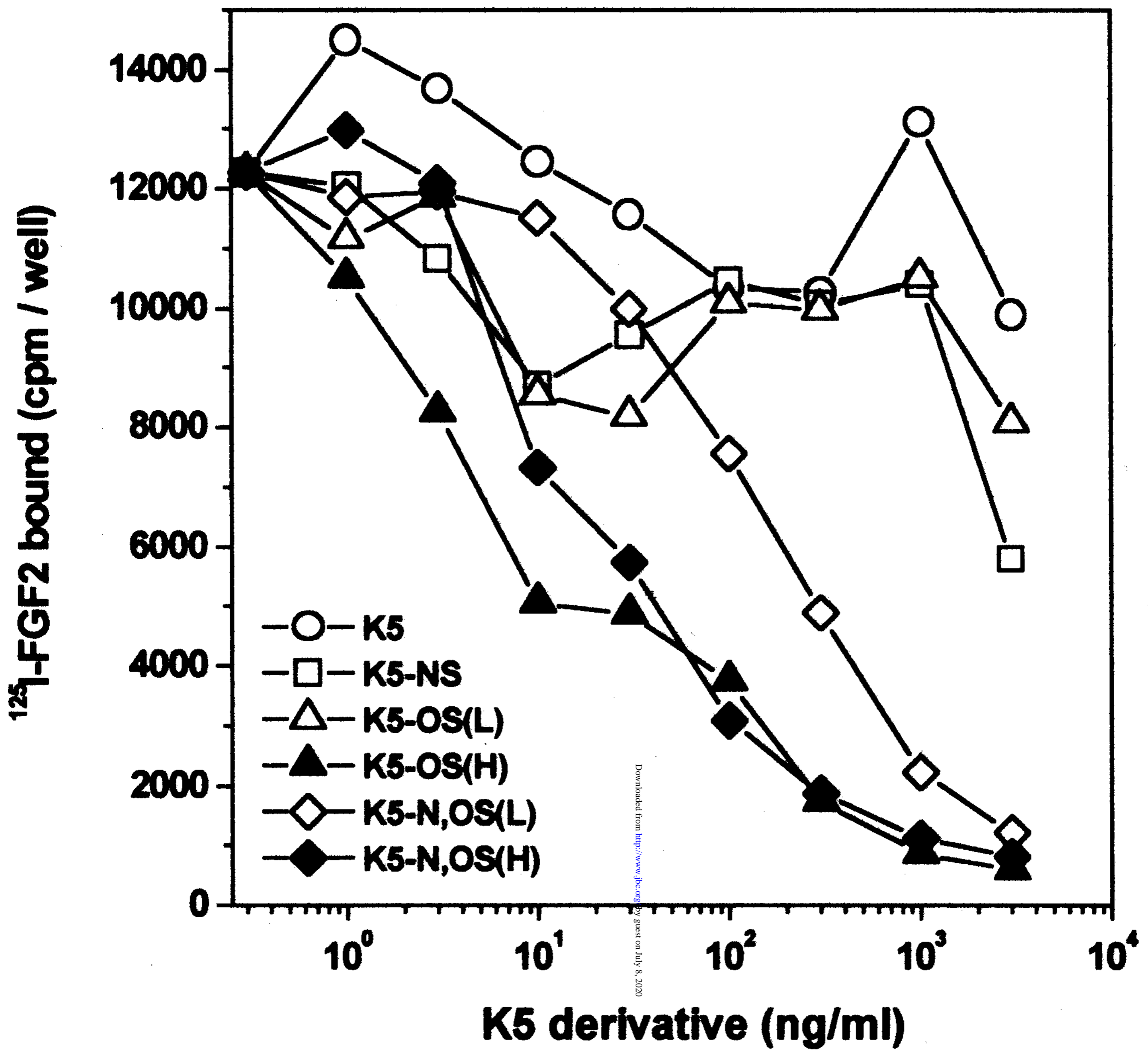


K5-N,OS(L)

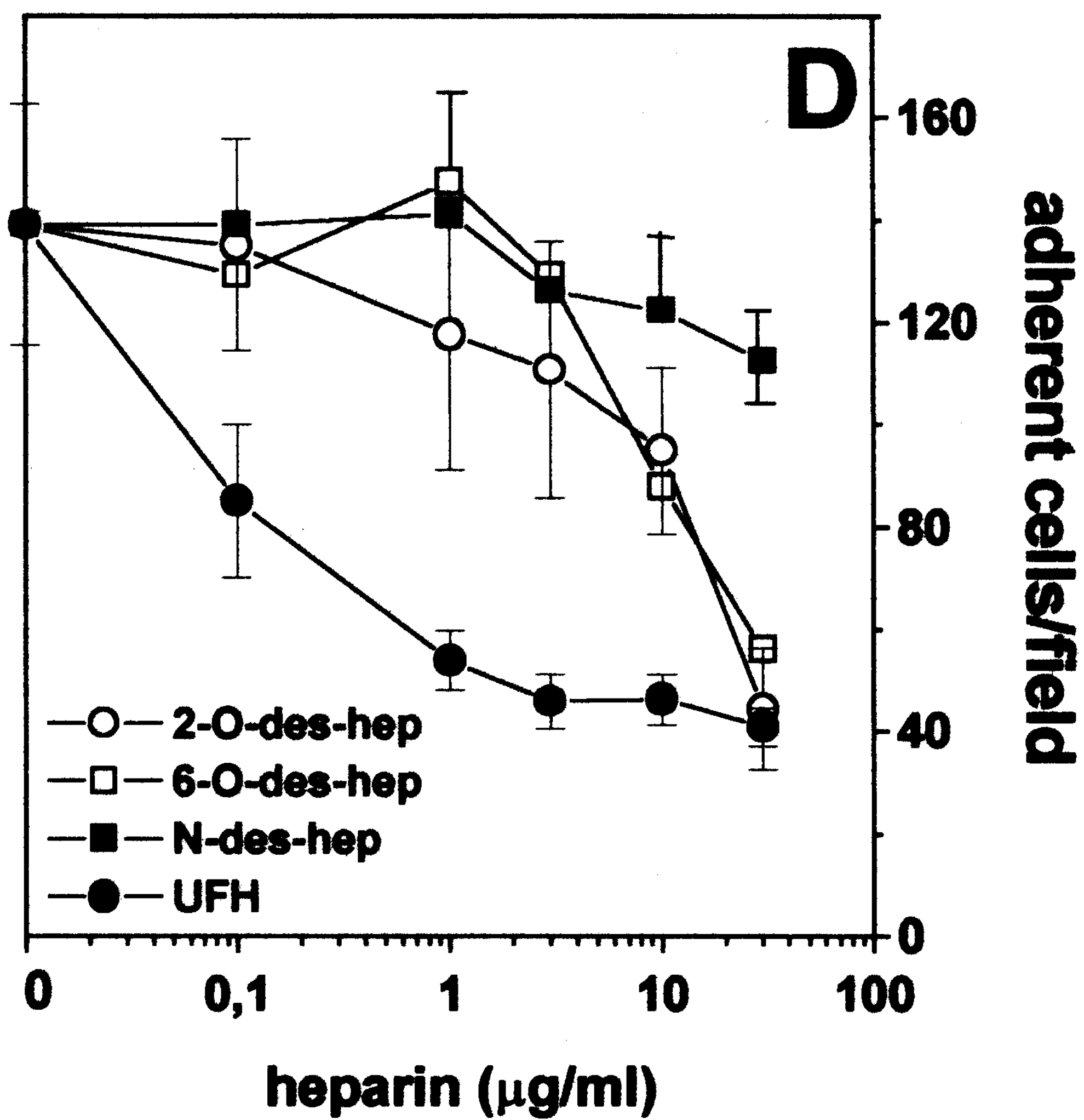
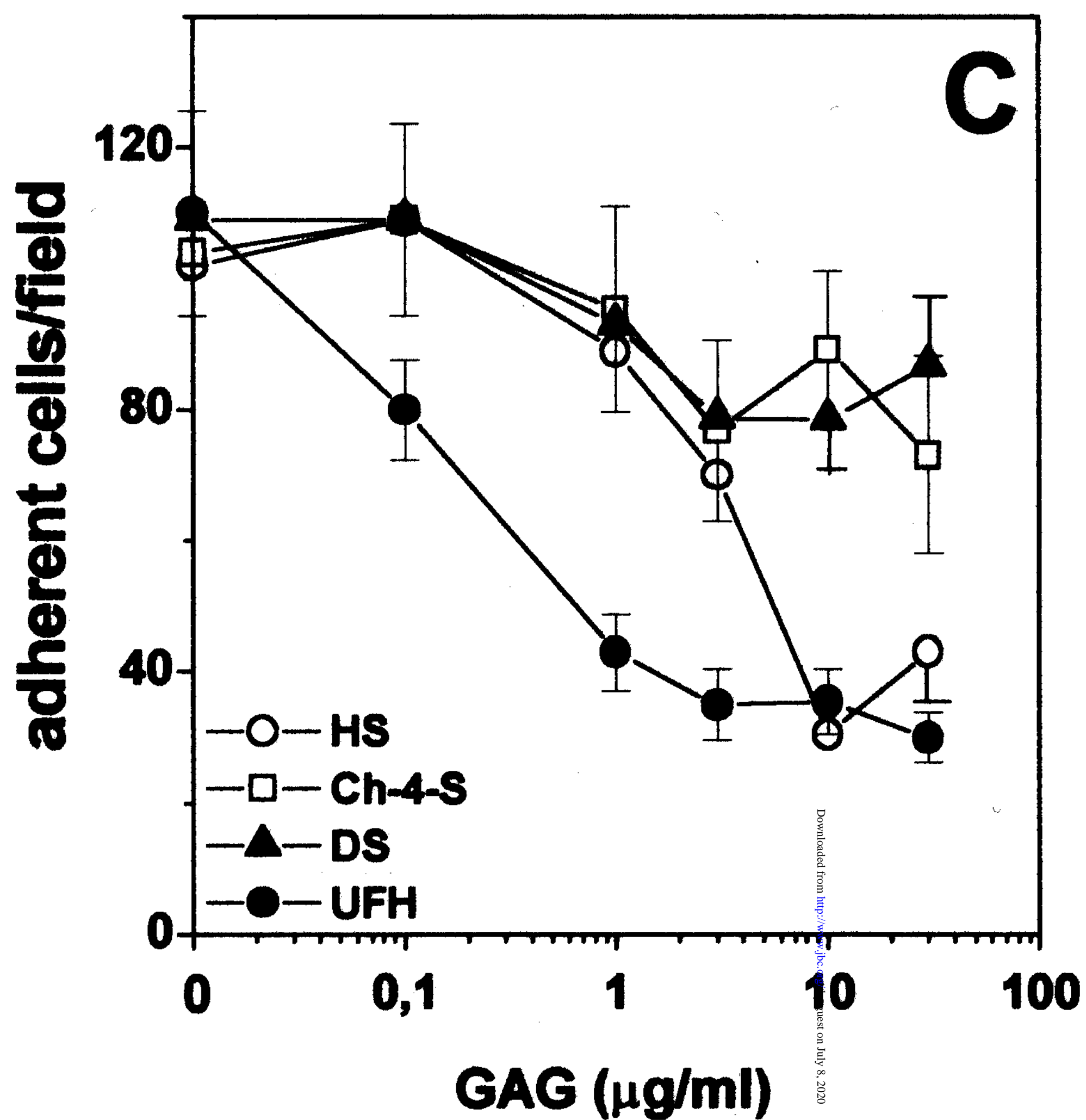
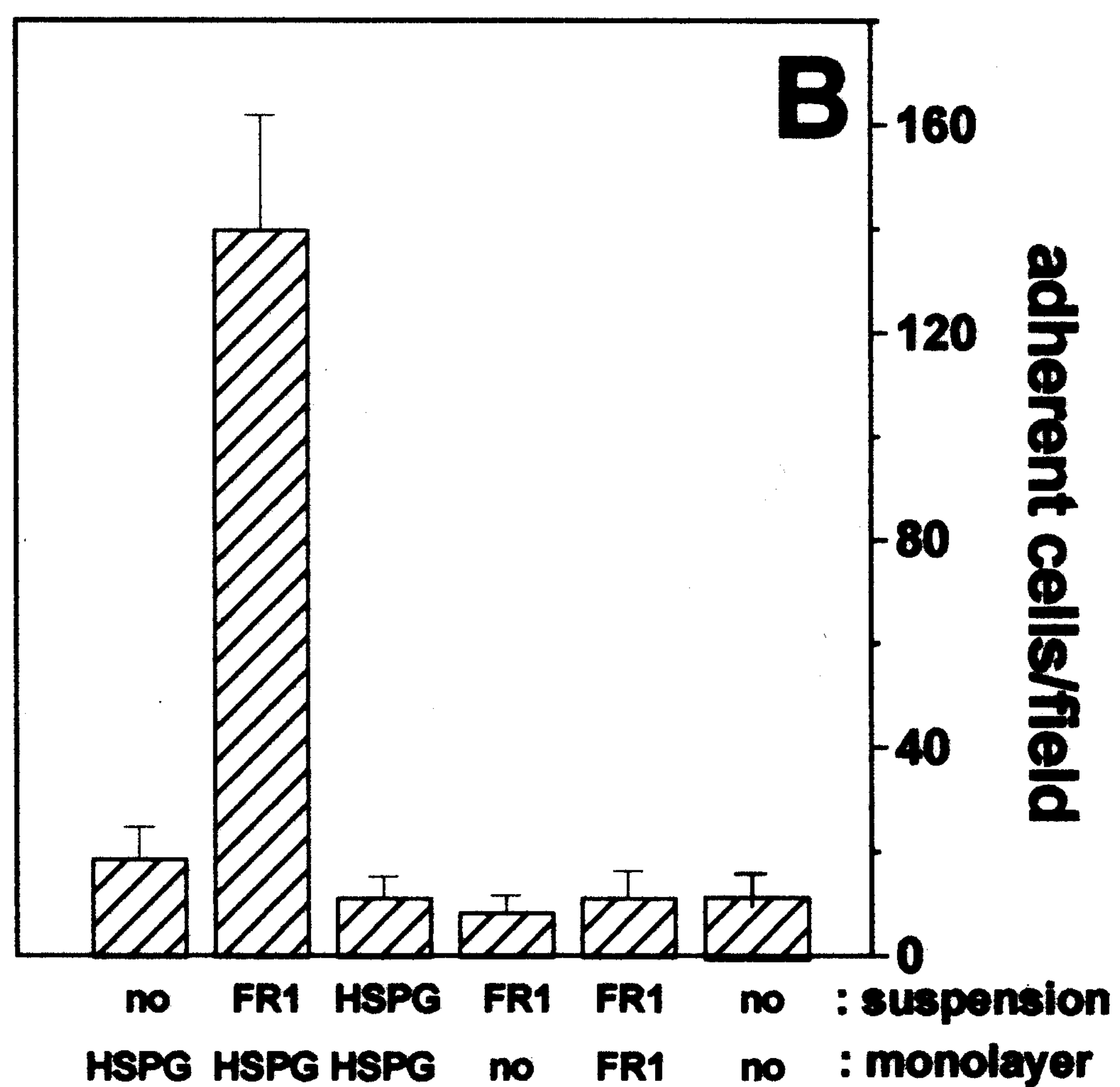
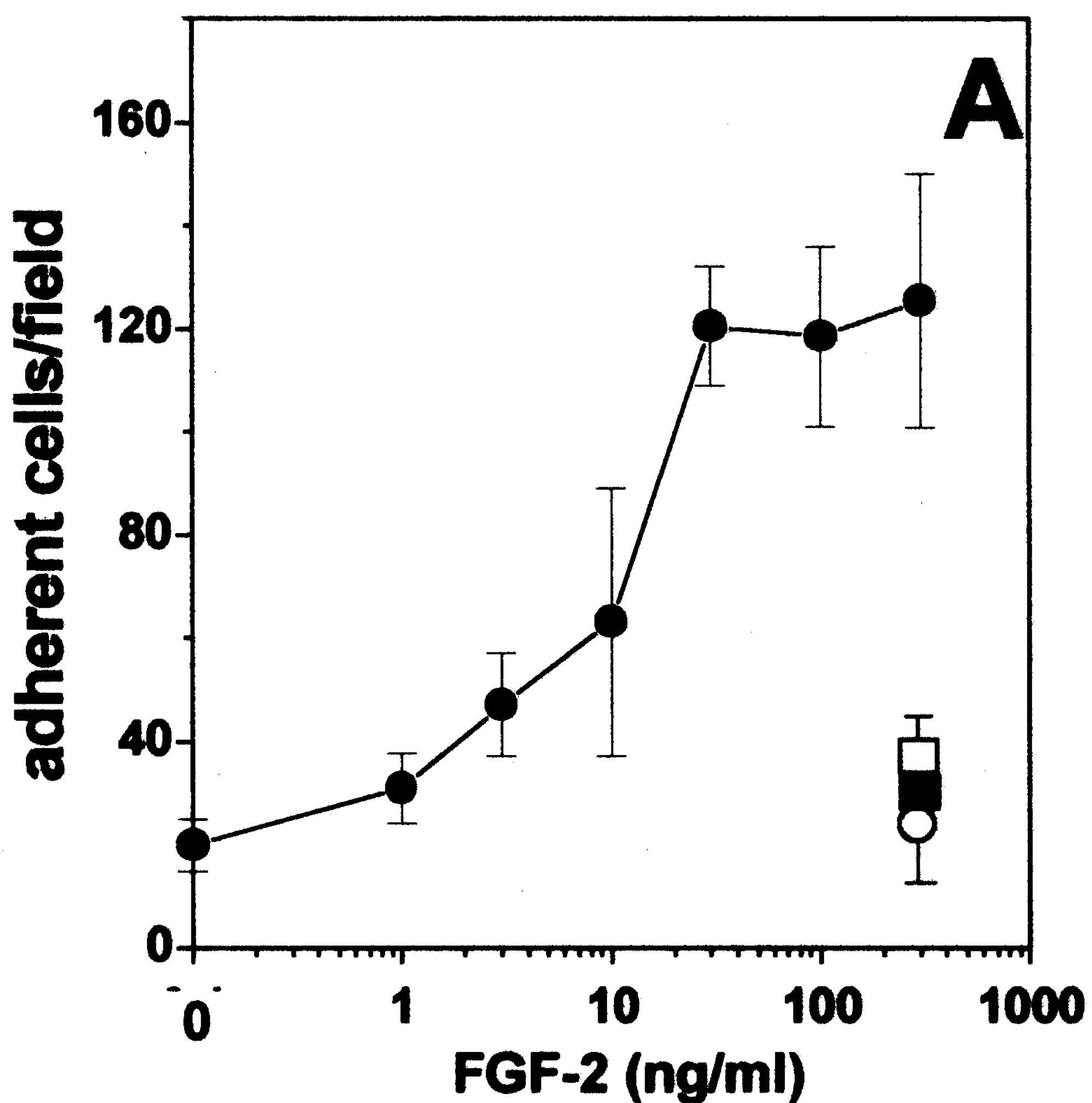


K5-N,OS(H)

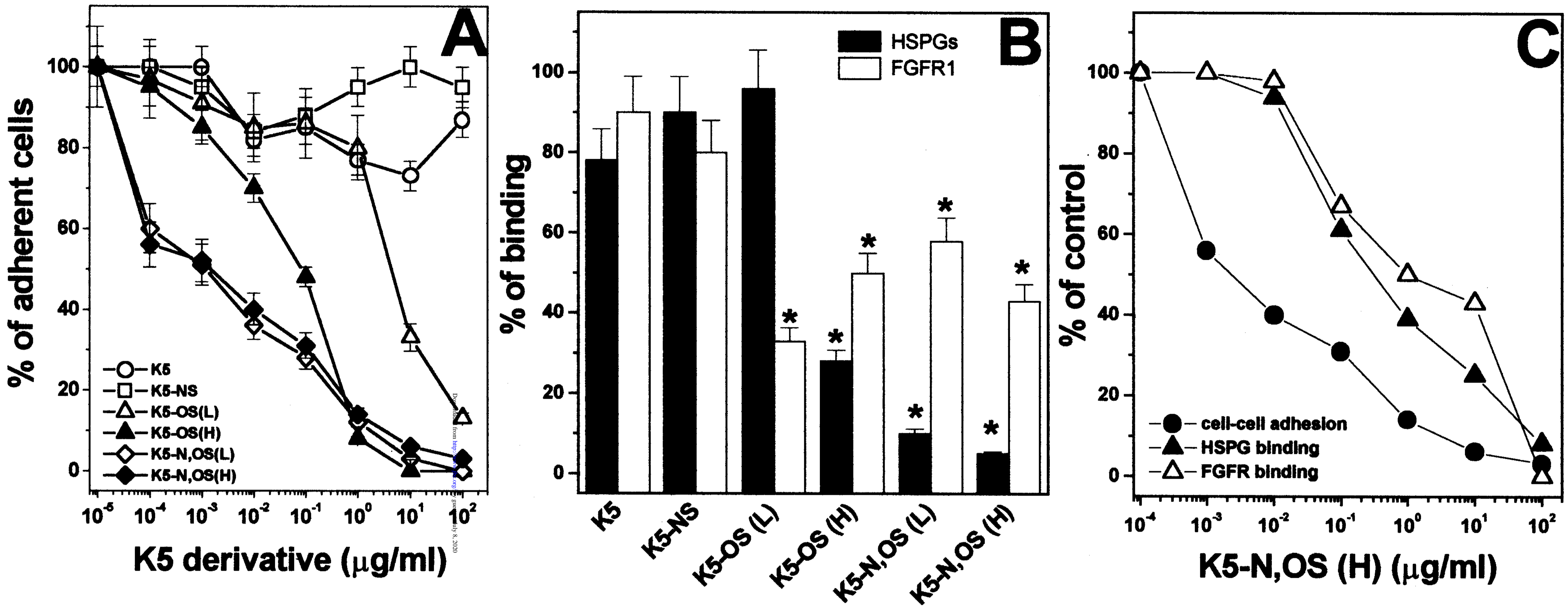
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 Inhibition of FGF2 activity by K5 derivatives
 Fig. 2

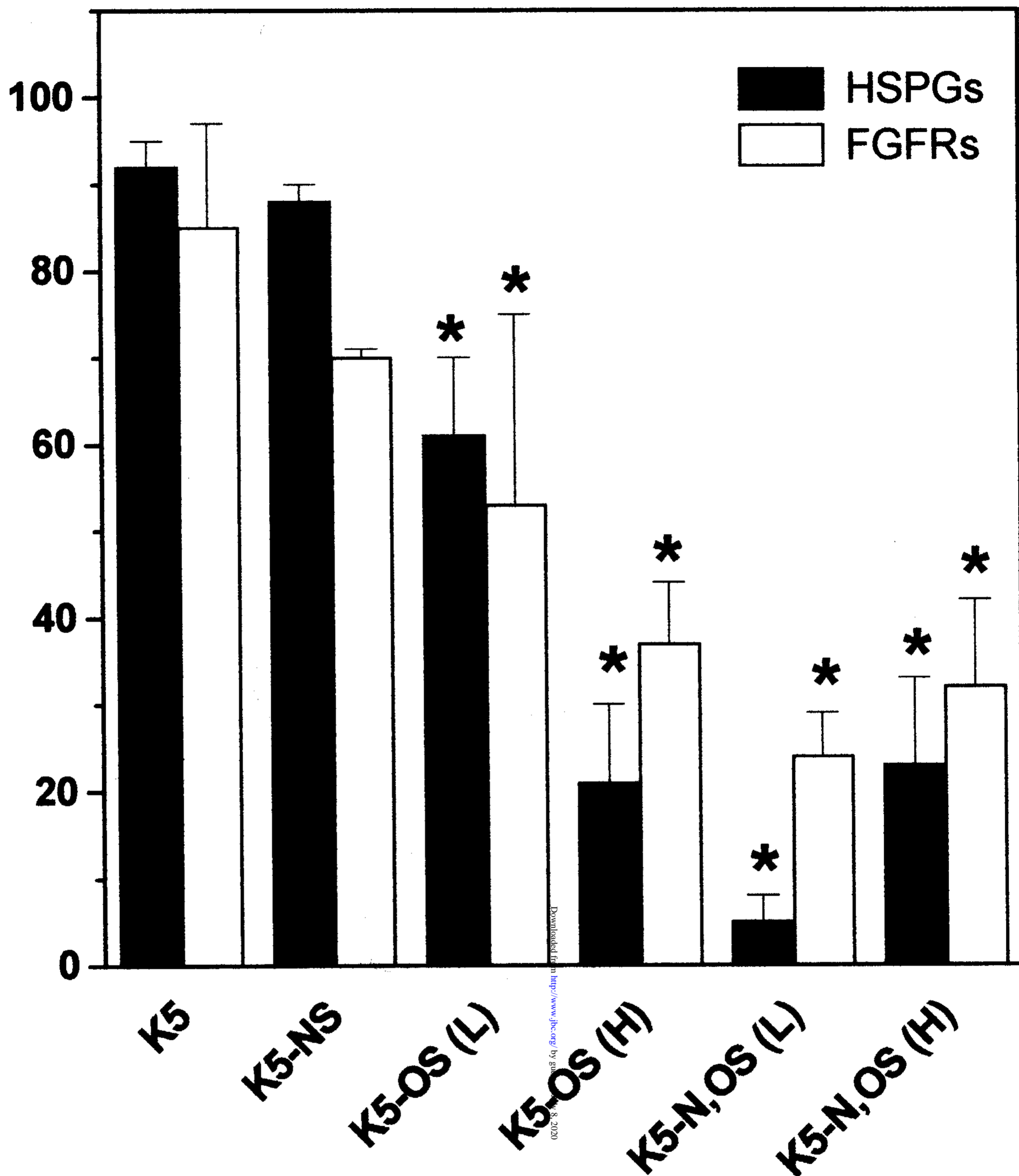


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 Inhibition of FGF2 activity by K5 derivatives
 Fig. 3

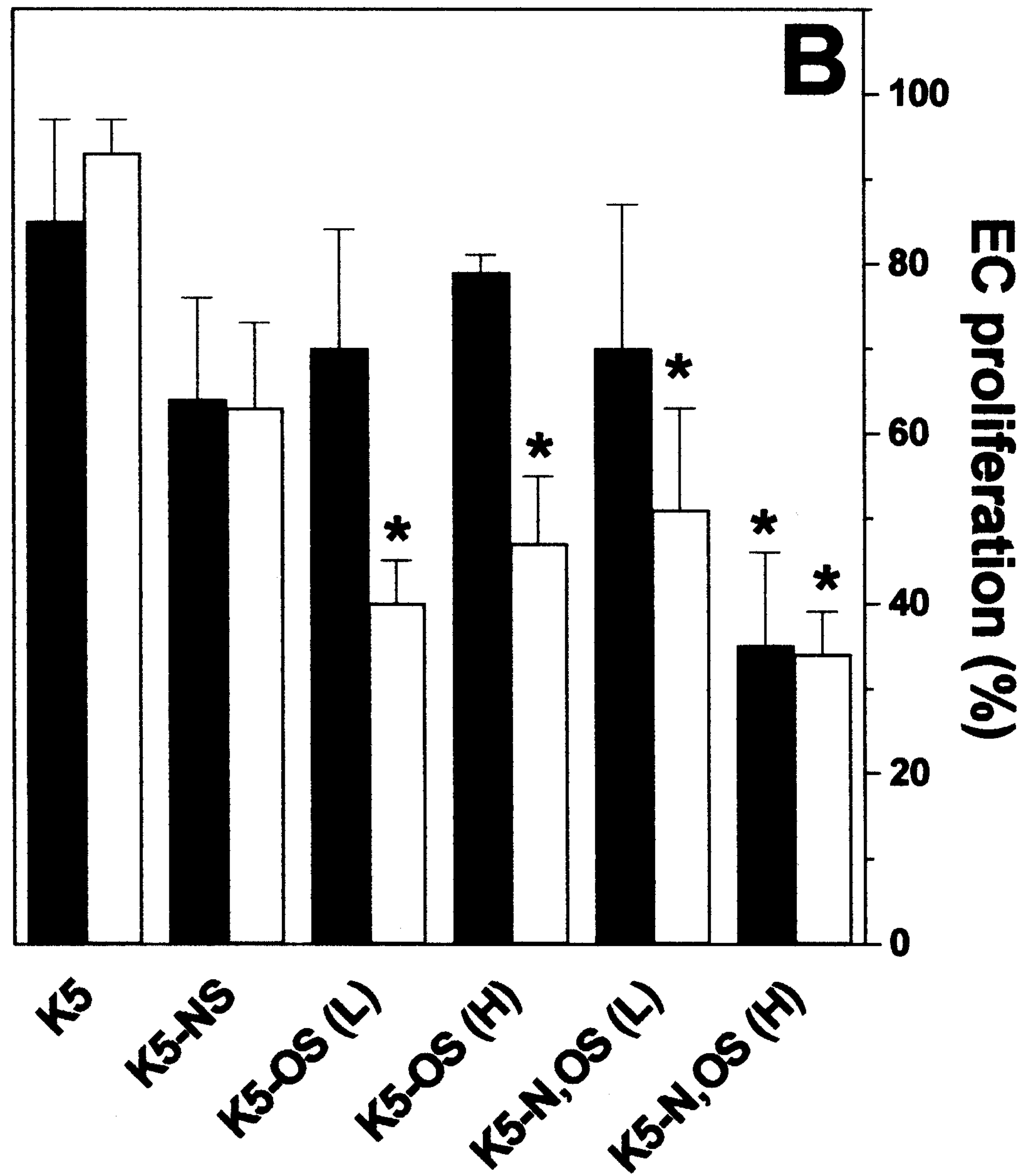
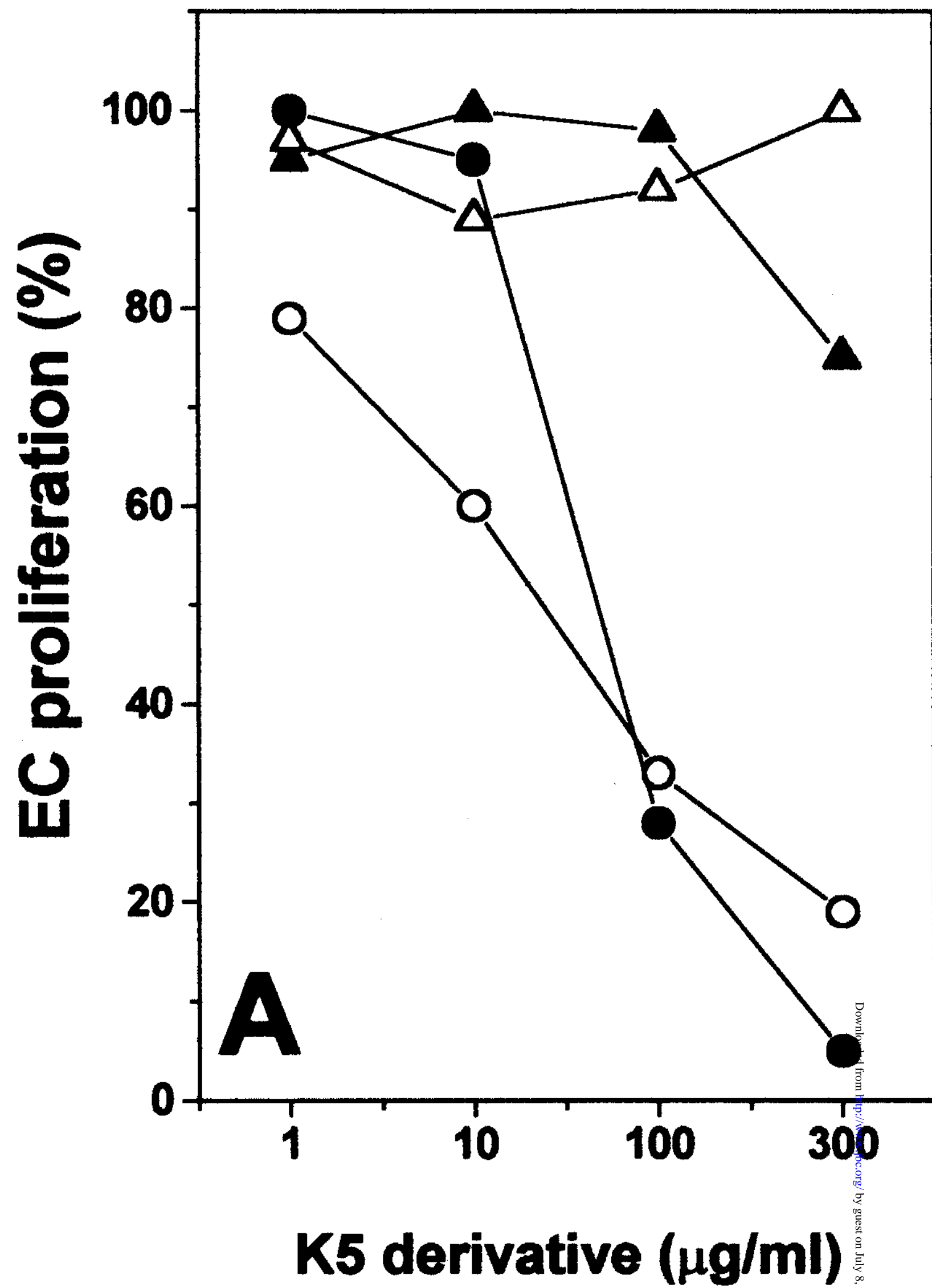


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 Inhibition of FGF2 activity by K5 derivatives
 Fig. 4

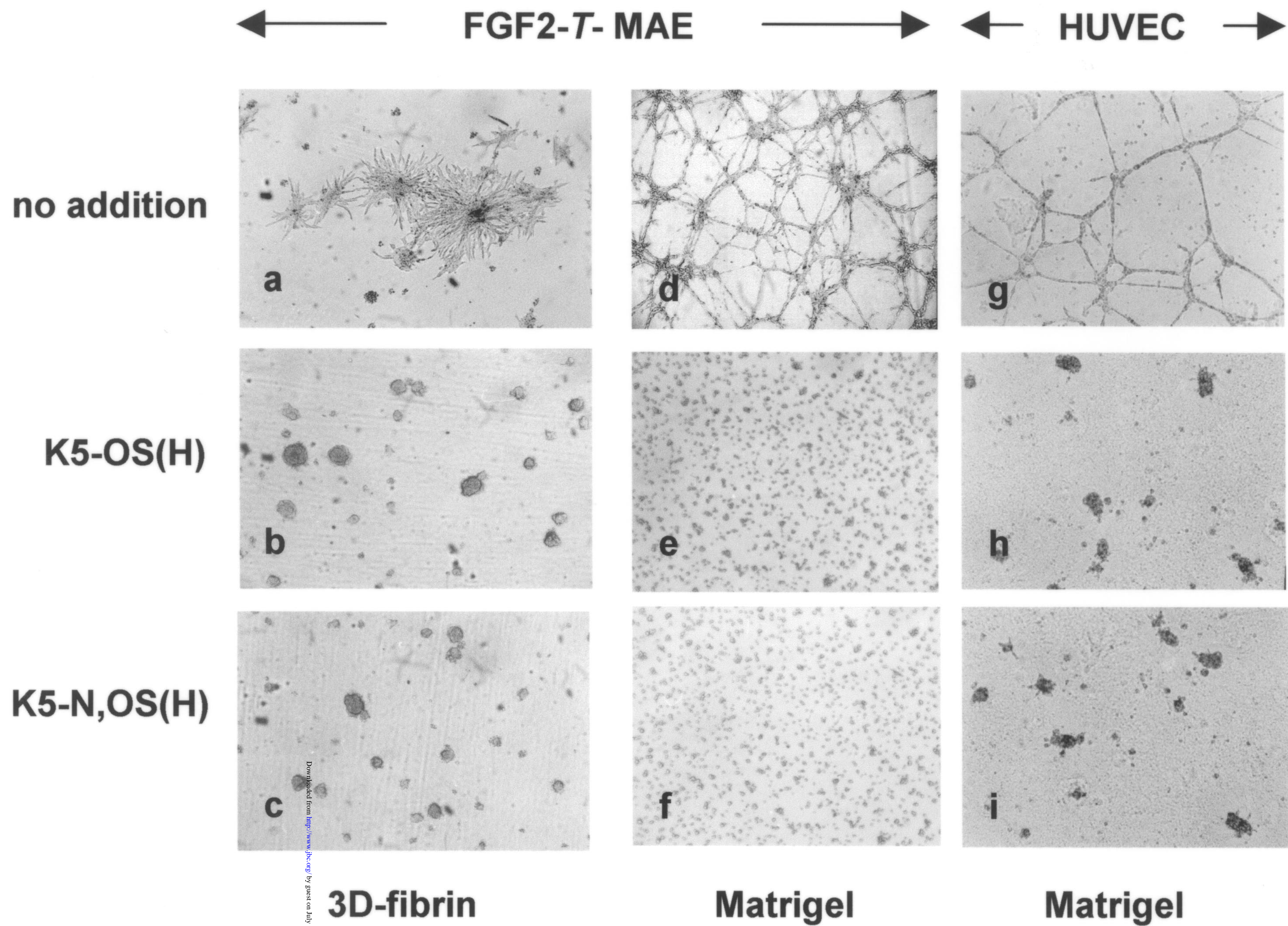
% of binding



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Inhibition of FGF2 activity by K5 derivatives
Fig. 5



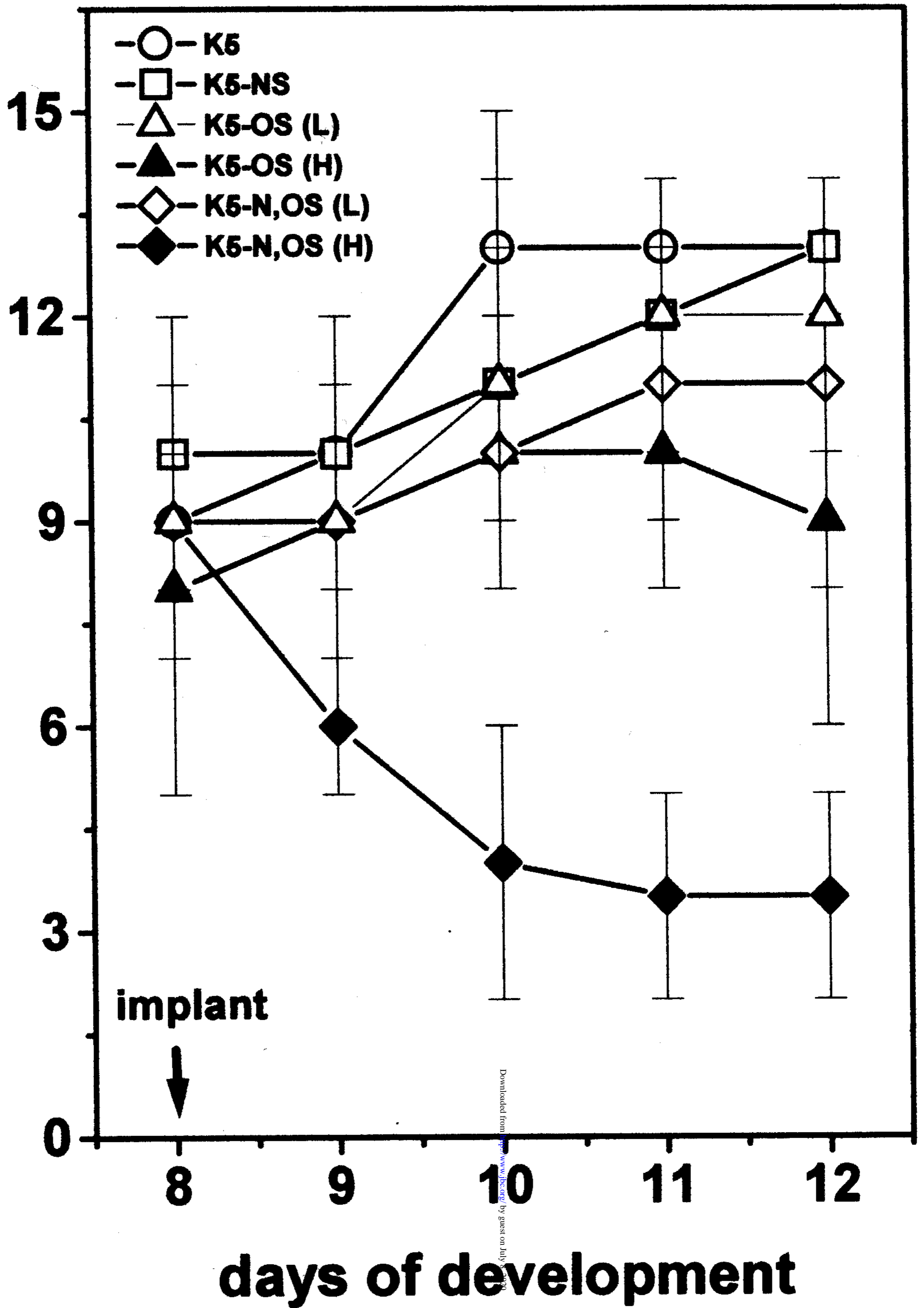
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 Inhibition of FGF2 activity by K5 derivatives
 Fig. 6



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Inhibition of FGF2 activity by K5 derivatives
Fig. 7

blood vessels/implant



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Inhibition of FGF2 activity by K5 derivatives
Fig. 8

**Fibroblast growth factor-2 antagonist activity and angiostatic capacity of sulfated
Escherichia coli K5 polysaccharide derivatives**

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