0026-895X/03/6403-696–702$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 64:696–702, 2003

Vol. 64, No. 3
2487/1084482
Printed in U.S.A.

Low Molecular Weight Fucoidan and Heparin Enhance the Basic Fibroblast Growth Factor-Induced Tube Formation of Endothelial Cells through Heparan Sulfate-Dependent α6 Overexpression

DELPHINE CHABUT, ANNE-MARIE FISCHER, SYLVIA COLLIEC-JOUAULT, INGRID LAURENDEAU, SABINE MATOU, BERNARD LE BONNIEC, and DOMINIQUE HELLEY

Institut National de la Santé et de la Recherche Médicale U428 (D.C., A.-M.F., B.L.B., D.H.) and Laboratoire de Génétique Moléculaire-Unité Propre de Recherche et de l'Enseignement Supérieur, Equipe d'Accueil 3618 (I.L.), Université Paris V, Paris, France; Hôpital Européen Georges Pompidou, Assistance Publique - Hôpitaux de Paris, Paris, France (A.-M.F., D.H.); and Institut Française de Recherche pour l'Exploitation de la Mer, Nantes, France (S.C.-J., S.M.);

Received February 27, 2003; accepted May 16, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Basic fibroblast growth factor (FGF-2) activates its high-affinity receptors (FGFRs) but also acts through interaction with heparan sulfate proteoglycans (HSPG). Exogenous polysaccharides also modulate the angiogenic activity of FGF-2. We investigated the effect and mechanism of action of a low molecular weight fucoidan derivative (LMWF) on tube formation by human endothelial cells. LMWF has a better arterial antithrombotic potential in animals than low molecular weight heparin (LMWH). After stimulation of human umbilical vein endothelial cells (HUVEC) by FGF-2 and LMWF (or LMWH), we observed 1) using flow cytometry, an increase in the amount of the α6 integrin subunit; 2) using quantitative reverse transcription-polymerase chain reaction, an increase in α6 mRNA (higher with LMWF than with LMWH); and 3) using a Matrigel model, an increase in vascular tube formation (also higher with LMWF than with LMWH). A direct link between α6 overexpression and vascular tube formation was confirmed by use of an anti-α6 antibody: in its presence, there was no capillary network formation on Matrigel. Unexpectedly, an anti-FGFR blocking antibody had no effect on α6 over-expression, whereas stripping off the heparan sulfate with heparitinases abolished overexpression. Overall, our data suggest that FGF-2 stimulates α6 over-expression in HUVEC, through HSPG but independently from FGFR, and that LMWF (or LMWH) modulates this interaction. Expression of heparan sulfate proteoglycan increases after ischemic injury. Given its antithrombotic properties and its ability to potentiate tube formation of endothelial cells, LMWF may have to be considered for revascularization of ischemic areas.

Development of appropriate strategies to induce neovascularization after vessel wall injury (ischemia, angioplasty) is at the forefront of biomedical research. Models that mimic angiogenesis, however, are still needed to identify mechanisms and to provide tools allowing assessment of the efficacy of interfering compounds.

Basic fibroblast growth factor (FGF-2) is a member of the heparin-binding growth-factors family. It is involved in the control of cell growth, survival, and differentiation. Physiological processes using FGF-2 include tissue wound repair and neovascularization. In vivo models support the idea that FGF-2 induces neovascularization (Ribatti et al., 1995) and growth of new blood vessels after vascular injury (Yanagisawa-Miwa et al., 1992). In vitro models confirm that FGF-2 induces endothelial cell proliferation and migration and alters expression and/or activation of integrins (Moscatelli et al., 1986; Klein et al., 1993). The biological functions of FGF-2 are mediated through interactions with high- and low-affinity cell surface receptors. High-affinity FGF-2 receptors (FGFRs) have typical intracellular tyrosine-kinase activity (Fantl et al., 1993); low-affinity FGF-2 receptors involve the polysaccharides carried by heparan sulfate proteoglycans (HSPG) (Rifkin and Moscatelli, 1989; Rosenberg et al., 1997). Undoubtedly, the angiogenic effect of FGF-2 on vascular endothelial cells is mediated in part through modulation of its integrin-dependent adhesion (Diamond and Springer, 1994). Succinctly stated, high levels of α6 subunit are essential for the FGF-2–induced tubular morphogenesis of human umbilical vein endothelial cells (HUVEC) (Bauer et al., 1992; Matou et al., 2002). High levels of α6 subunit also seem to be

ABBREVIATIONS: FGF-2, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; HSPG, heparan sulfate proteoglycans; HUVEC, human umbilical vein endothelial cells; LMWF, low molecular weight fucoidan; LMWH, low molecular weight heparin; FCS, fetal calf serum; RT-PCR, reverse transcription-polymerase chain reaction; Ct, cycle threshold.

associated with tumor cells in prostate cancer as well as hepatocarcinomas (Rabinovitz et al., 1995; Carloni et al., 1998). The α6 subunit combines with the β1 subunit to form the laminin receptor, one of the main constituents of the basal membrane. Other integrins, such as $\alpha v\beta 3$, also seem to play a crucial role in the acquisition of angiogenic properties by endothelial cells (Yeh et al., 1999). Heparan sulfates, on the other hand, protect FGF-2 from proteolysis and degradation (Saksela et al., 1988), thus serving as reservoirs that release FGF-2 upon proteoglycan degradation. HSPG also promote FGF-2 binding to FGFR and its subsequent activation (Yayon et al., 1991). In addition to this FGFR-mediated pathway, HSPG induce internalization of FGF-2 in Chinese hamster ovary cells, as reported by Rhogani and Moscatelli (1992). Thus, the function of FGF-2 on endothelial cells depends in part upon the availability of extracellular glycosaminoglycans, suggesting that exogenous polysaccharides could modulate the angiogenic response. Indeed, unfractionated heparin (Yayon et al., 1991), as well as heparin-mimicking compounds such as synthetic sulfonic acid polymers (Liekens et al., 1999), sulfated dextrin (Thornton et al., 1999), suramin analogs (Firsching et al., 1995), pentosan polysulfate (Zugmaier et al., 1992), and sulfated chitin derivates (Murata et al., 1991), inhibit the proangiogenic activity of FGF-2. Overall, unfractionated heparin and mimicking compounds seem to act by competition with HSPG for the binding of FGF-2, thus hampering formation of the HSPG/FGF-2/FGFR ternary complex.

Fucoidans are high molecular weight sulfated polysaccharides of marine plant origin (brown seaweed *Ascophyllum nodosum*). Reduced into low molecular weight fractions, fucoidans are suitable for in vivo experiments. In a previous study (Matou et al., 2002), we reported that, in contrast to unfractionated heparin, a 16-kDa fucoidan subfraction allows HUVEC to differentiate upon addition of FGF-2. In this article, we compared the effect of a 4-kDa fucoidan subfraction (LMWF) with that of a 5-kDa low molecular mass heparin (LMWH) on the FGF-2–induced tube formation of HUVEC. To this end, we have quantified the influence of these polysaccharides on the expression of α6 and β1 integrin subunits. We first demonstrated that LMWF and LMWH potentiated, through overexpression of the α6 integrin, the FGF-2–induced tubular morphogenesis. To further identify the mechanism of LMWF and LMWH modulation, we evaluated the contribution of HSPG and FGFR on α6 overexpression. Over-expression was abolished by heparitinases, whereas an anti-FGFR blocking antibody was without effect. Taken together, our data evidenced a novel, cooperative effect between LMWF (or LMWH) and HSPG in response to FGF-2.

Experimental Procedures

Materials. LMWF (4 kDa), isolated and fractionated as described by Nardella et al. (1996), was a gift from Institut Française de Recherche pour l'Exploitation de la Mer (Nantes, France). Unfractionated heparin (average molecular mass, 16 kDa) was from Sanofi Center (Toulouse, France), and LMWH (Dalteparin, 5 kDa) was from Pharmacia (Guyancourt, France). Human recombinant FGF-2 was purchased from Valbiotech (Paris, France), and growth factor-reduced Matrigel was from BD Biosciences (Le Pont de Claix, France). Rat anti-human α6 subunit (CD49f) monoclonal antibody conjugated to phycoerythrin was from BD Biosciences Pharmingen (Le Pont de Claix, France), and mouse anti-human β1 subunit (CD29) monoclonal antibody conjugated to phycoerythrin was from Caltag (le Peray en Yvelines, France). Anti-FGF-2, anti-FGFR, and anti-α6 subunit blocking antibodies, together with the corresponding irrelevant isotype matched antibodies, were from Chemicon International (Souffelweyersheim, France). Heparitinase *Flavobacterium heparinum* I, II, and III were from Calbiochem (Fontenay-ss-Bois, France).

Cell Culture and Treatment. HUVEC were isolated from two to five human umbilical cords according to the method described by Giraux et al. (1998). At third passage, HUVEC were seeded at 1.5×10^5 cells/ml in culture medium (50% Med 199 and 50% RPMI 1640 medium; Invitrogen, Cergy-Pontoise, France) supplemented with 20% fetal calf serum (FCS) onto plates coated with 0.5% gelatin. After 24 h, cells were fed with medium supplemented with 5% FCS, with or without sulfated polysaccharides (0.1 to 10 μg/ml) and/or FGF-2 (5 ng/ml). Medium was renewed 48 h later, and HUVEC were detached 24 h later using versene containing 0.01% collagenase. Cells were centrifuged at 200*g* for 8 min at 4°C and were washed twice in cooled Hank's solution containing 2% FCS. Cells were kept in this buffer until use in flow cytometry analysis or in vascular tube formation assays. Part of HUVEC was treated beforehand with heparitinase I, II, and III (0.01 U/ml each in phosphate-buffered saline) for 1 h at 37°C and were retreated along each feeding. Part of HUVEC was grown in the presence of 10 μg/ml blocking FGFR antibody (to prevent FGF-2 binding). In control experiments, HUVEC were cultured in the presence of 10 μg/ml blocking FGF-2 antibody.

Tube Formation. HUVEC were resuspended in culture medium supplemented with 5% FCS and seeded onto Matrigel (6×10^4 cells/ml). Before inoculation onto Matrigel, part of HUVEC were resuspended in medium containing 10 μg/ml anti-α6 blocking antibody or, in control experiments, in medium containing 10 μg/ml of the corresponding irrelevant isotype-matched antibody. Matrigel plates were incubated at 37°C in humidified atmosphere containing 5% CO_2 for 18 h. Cells in developed Matrigel were fixed in 1.1% glutaraldehyde, and analyzed for tube formation by phase-contrast microscopy.

Flow Cytometry Analysis. HUVEC were incubated for 30 min with the anti-α6 conjugated antibody, alternatively with the anti-β1 conjugated antibody. The surface labeling of each integrin subunit was quantified by immunofluorescence on a FACSCalibur flow cytometer (BD Biosciences).

Real-Time RT-PCR. Total RNA was extracted from HUVEC by standard method using acid-phenol guanidinium (RNAble; Eurobio, les Ulys, France) and was reverse-transcribed before real-time PCR. The primers used were 5′-CACATCTCCTCCCTGAGCACAT-3′ (ITGA6-U) and 5′-TATATCTTGCCACCCATCCTTGTT-3′ (ITGA6-L), giving a 104-base pair product representing the *ITGA6* gene expression (i.e., α6 mRNA), and 5′-TGCACAGGAGCCAAGAGTGAA-3′ (TBP-U) and 5′-CACATCACAGCTCCCCACCA-3′ (TBP-L), giving a 132-base pair product representing the transcript of the *TBP* gene coding for the TATA box-binding protein. The latter transcript was used as endogenous control to estimate the amount and integrity of the total RNA in each reaction. PCR was performed using the SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min followed by 50 cycles comprising a 95°C step for 15 s and a 65°C step for 1 min. Amount of mRNA was evaluated from the number of cycles (Ct) that was necessary to reach an increase in fluorescence associated with an exponential growth of the PCR products. Ct was estimated in duplicate, using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Results are expressed as -fold differences in *ITGA6* gene expression relative to that of *TBP* using the formula: N*ITGA6* = $2\Delta^{Ct}$, where N*ITGA6* is the -fold difference in *ITGA6* gene expression, and ΔCt is the difference between the average Ct value for the *ITGA6* expression and the average Ct value for the *TBP* gene.

Statistical Analysis. Data are expressed as mean ± S.E. Significant differences were determined using analysis of variance followed by a Fisher's protected least-significant difference test ($p < 0.05$).

Results

Linkage between Tubular Morphogenesis and α6 Expression in FGF-2–Stimulated HUVEC. HUVEC seeded on a standard in vitro Matrigel system mimics both endothelial cell migration and differentiation and thus constitutes a suitable model to explore tubular morphogenesis. Indeed, 18 h after seeding on Matrigel, HUVEC pretreated with 5 ng/ml FGF-2 differentiated in a partially organized capillary network, whereas untreated cells were unable to form vascular tubes (Fig. 1A). Cell migration and differentiation depend on specific interactions between integrin(s) of endothelial cells and component(s) of the extracellular matrix. The most abundant component of Matrigel is laminin, which binds its receptor on HUVEC constituted by the association of α6 and β1 subunits. We first investigated whether FGF-2 modulated α6 and/or β1 expression. Flow cytometry confirmed that, compared with untreated cells, FGF-2–stimulated HUVEC exhibited a 3-fold increase ($p < 0.01$) of the α6 cell-surface labeling, whereas no increase could be evidenced with respect to β1 labeling (Fig. 1B). To further explore the increase in α6 expression, we quantified its mRNA by real-time RT-PCR. After FGF-2 stimulation, the α6 integrin mRNA represented 3-fold more than that of untreated cells ($p < 0.05$) (Fig. 1C). We then investigated whether vascular tube formation could be linked to α6 overexpression by adding a blocking anti-α6 antibody to FGF-2–stimulated HUVEC. Tubular morphogenesis on Matrigel was totally blocked in the presence of the antibody, whereas enhanced vascular tube formation still occurred when using an irrelevant isotype-matched antibody (Fig. 2A).

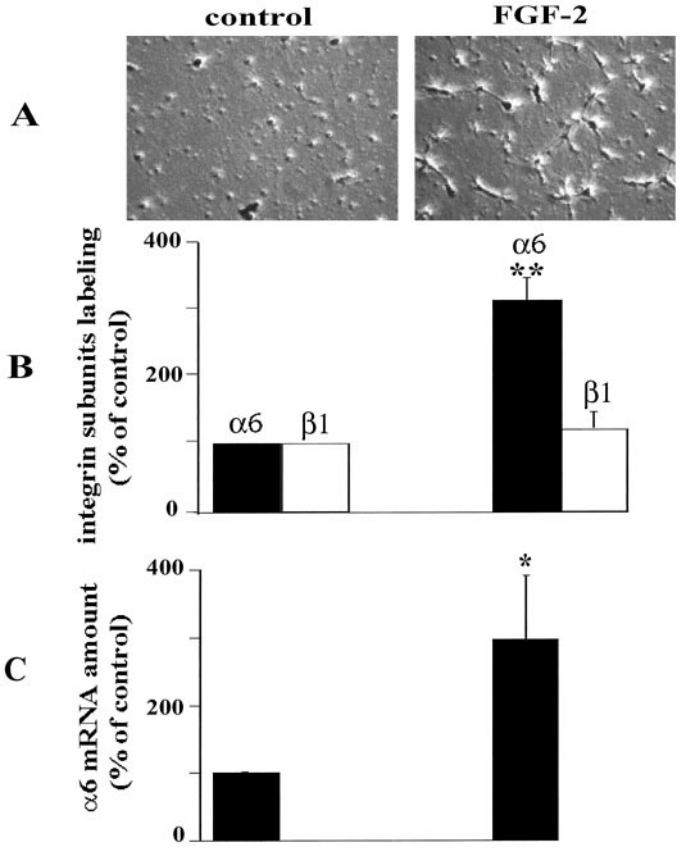

Fig. 1. Overexpression of α6 integrin is required for vascular tube formation mediated by FGF-2. HUVEC were grown for 72 h in medium supplemented with 5% FCS only (left) or containing 5 ng/ml FGF-2 (right). A, effect of FGF-2 on vascular tube formation. Cells were seeded onto Matrigel and analyzed by phase contrast microscopy 18 h after seeding. Photographs are representative of a minimum of three experiments. B, effect of FGF-2 on cell surface labeling of α6 and β1 integrin subunits quantified by flow cytometry. Data are expressed as percentage of the mean fluorescence compared with untreated cells (mean ± S.E. of seven experiments). C, effect of FGF-2 on the amount of α6 mRNA transcripts quantified by real time RT-PCR. Data are expressed as a percentage of the amount of α6 transcript compared with untreated cells (mean ± S.E. of three experiments in duplicate). *, $p < 0.05$, **, $p < 0.01$, significantly different from control.

LMWF and LMWH Enhance FGF-2–Induced Tubular Morphogenesis and α6 Expression. Polysaccharides rich in sulfate and carboxylic groups bind FGF-2 and modulate its activity. We thus speculated that LMWF and/or LMWH might control the morphogenic potential of FGF-2 that we detected using Matrigel. HUVEC incubated simultaneously with LMWF (10 μg/ml) and FGF-2 (5 ng/ml) branched and formed capillary-like tubes that organized in closed areas, denoting intense differentiation. HUVEC stimulated with FGF-2 and LMWH (instead of LMWF) also formed capillary tubes with closed structures, albeit to a lower extent (Fig. 2A). Because the α6-mediated interaction of HUVEC with Matrigel played a crucial role in their ability to form vascular tubes, we reasoned that the enhanced FGF-2 effect observed with LMWF and LMWH could originate from a modulation of α6 and/or β1 expression. Flow cytometry revealed that incubation of HUVEC with FGF-2 and LMWF or LMWH resulted in a 2-fold increase of the α6 integrin cell-surface labeling, compared with cells stimulated by FGF-2 alone ($p < 0.001$ or $p < 0.05$, respectively; Fig. 2B). Quantitative RT-PCR indicated that incubation of HUVEC with FGF-2 and LMWF resulted in a 4-fold ($p < 0.001$) increase of the α6 mRNA amount, still compared with cells stimulated by FGF-2 only (Fig. 2C), whereas incubation of HUVEC with FGF-2 and LMWH resulted in a 2-fold ($p < 0.05$) increase of the α6 mRNA amount. With respect to the β1 integrin, flow cytometry uncovered only a small, yet detectable, enhancement after FGF-2 and LMWF (or LMWH) stimulation, again compared with the labeling obtained for cells stimulated by FGF-2 only (Fig. 2B). Consistent with the hypothesis that vascular tube formation was related to α6 overexpression, stimulating HUVEC with FGF-2 and LMWF (or LMWH) in the presence of a blocking anti-α6 antibody totally blocked tubular morphogenesis on Matrigel (Fig. 2A), whereas enhanced vascular formation still occurred when using the corresponding irrelevant isotype-matched antibody (Fig. 2A). Taken together, these observations suggested that LMWF and LMWH potentiated the FGF-2-induced α6 over-expression.

Cell-Surface Heparan Sulfates Are Required for FGF-2–Induced α6 Overexpression. It is well established that unfractionated heparin restores the biological effects of FGF-2 on heparan-sulfate depleted cells. The cellular responses to FGF-2 stimulation result from the interaction of FGF-2 with FGFR and/or HSPG. To assess whether cell-surface heparan sulfates participated in normal α6 expression, HUVEC were treated with heparitinases (I, II, and III;

10 mU/ml each) before FGF-2 stimulation, and the amount of α6 integrin was quantified (Fig. 3). Flow cytometry revealed that when heparan sulfate-depleted HUVEC were incubated in the presence of 5 ng/ml FGF-2 (Fig. 3B), α6 labeling remained comparable with that obtained with nonstimulated cells (Fig. 1B) or with cells cultured in the presence of an anti-FGF-2 antibody (Fig. 3D). That heparitinases abrogated the FGF-2 stimulation raised the question as to whether LMWF and/or LMWH would (as unfractionated heparin) substitute for cell-surface heparan sulfate. To test this hypothesis, heparan sulfate-depleted HUVEC were simultaneously incubated with FGF-2 and LMWF (or LMWH), and the amount of α6 on cells was quantified. Compared with FGF-2–stimulated cells bearing heparan sulfates, the amount of α6 was markedly reduced with heparan sulfate-depleted HUVEC, whether or not LMWF (or LMWH) was present (Fig. 3B). However, although greatly reduced, α6 expression remained significantly higher ($p < 0.01$) in HUVEC incubated in the presence of LMWF (Fig. 3B). As above, α6 expression was actually comparable with that obtained with cells grown in the presence of an anti-FGF-2 antibody (Fig. 3D). The same was not true with heparan-sulfate depleted HUVEC incubated in the presence of LMWH, even if, in our system too, unfractionated heparin preserved the FGF-2 stimulation of heparan sulfate depleted HUVEC (Fig. 3B). Thus, neither LMWF nor LMWH could substitute for unfractionated heparin to restore the FGF-2 stimulation of heparan sulfate-depleted HUVEC. Although surprising at first, this observation could be related to the properties of unfractionated heparin, having rather an inhibitory effect, if any, on vascular tube formation. Considering that cell-surface heparan sulfate promoted binding of FGF-2 to FGFR, we wondered about the precise role of FGFR in this system. Surprisingly, the anti-FGFR blocking antibody was devoid of detectable influence on FGF-2-induced α6 expression, even with simultaneous addition of LMWF or LMWH (Fig. 3C). Thus, this peculiar mode of action of FGF-2 seemed independent of FGFR; it apparently relied exclusively upon the presence of HSPG.

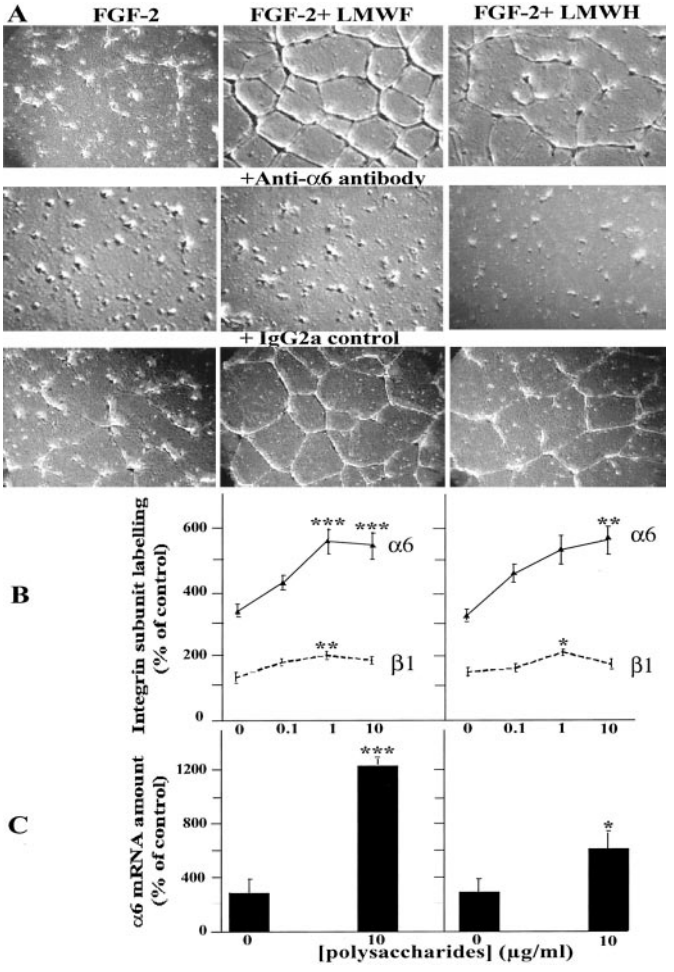

Fig. 2. LMWF and LMWH enhance the FGF-2–mediated overexpression of α6 integrin. A, HUVEC were grown for 72 h in medium supplemented with 5% FCS containing 5 ng/ml FGF-2 (left), FGF-2 and 10 μg/ml LMWF (middle), or FGF-2 and 10 μg/ml LMWH (right). Cells were seeded onto Matrigel and analyzed by phase contrast microscopy 18 h after seeding (top row). Some HUVEC were incubated with 10 μg/ml anti-α6 antibody (middle row) or the corresponding irrelevant antibody (bottom row). Photographs are representative of a minimum of three experiments. B, cells incubated in the presence of variable amounts of polysaccharides (as indicated) were analyzed by flow cytometry for surface labeling of the α6 and β1 subunits. Data are expressed as a percentage of the mean fluorescence intensity (mean ± S.E. of seven experiments) of untreated HUVEC as a function of the amount of polysaccharides added. C, cells incubated with or without 10 μg/ml polysaccharides (as indicated) were analyzed by real time RT-PCR for α6 mRNA transcripts. Data are expressed as percentage of the amount of α6 transcript compared with untreated cells (mean ± S.E. of three experiments in duplicate). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, significantly different from control.

LMWF Also Stimulates α6 Integrin Expression Independently of FGF-2. Neither the suppression of FGF-2 stimulation by using blocking anti-FGF-2 antibody nor the depletion of cell-surface heparan sulfates could totally quench the small (1.5-fold) yet significant ($p < 0.001$) LMWF-induced expression of α6 (Fig. 3, B and D). Thus LMWF seemed to enhance α6 integrin expression, independently of FGF-2. To challenge this pathway, we simply compared α6 expression of HUVEC stimulated with LMWF (without FGF-2) with that of cells cultured in the absence of stimulating factor (Fig. 4, B and C). After 72-h stimulation with 10 μg/ml LMWF, flow cytometry revealed that α6 integrin labeling increased 1.7-fold ($p < 0.001$) compared with nonstimulated cells (Fig. 4B). In agreement with a LMWF-specific, FGF-2-independent stimulation pathway, the amount of α6 mRNA also increased (2.1-fold; $p < 0.05$) compared with nonstimulated cells (Fig. 4C). LMWH could not substitute for LMWF: no increase in α6 labeling could be found when cells were incubated in the presence of LMWH. Despite the α6 increase, LMWF alone was unable to trigger tube formation on Matrigel (Fig. 4A).

Discussion

Finding compounds that interfere with the FGF-2 cellular responses would open perspectives in the development of new therapeutic agents. Specifically, numerous biological effects result from the interaction of FGF-2 with sulfated polysaccharides.

The present study illustrates that in a standard in vitro model (Matrigel), LMWF or LMWH enhanced the tubular morphogenesis induced by FGF-2. Upon stimulation, HUVEC branched and formed numerous tubes with closed areas. This is in contrast to stimulation by FGF-2 alone, triggering formation of a partial network only. Consistent with our results, a 16-kDa fucoidan fraction potentiates the FGF-2-induced angiogenesis of HUVEC (Matou et al., 2002).

Soeda et al. (2000) established, however, that a high molecular weight fucoidan has no effect. Discrepancy probably originates from the molecular weight of the fucoidan used. In fact, other studies pointed out that high molecular weight hyaluronans hamper tubular morphogenesis, whereas hyaluronan oligosaccharides stimulate endothelial cell proliferation and/or tube formation (Rahmanian et al., 1997). Depending upon its size, conflicting results on angiogenesis modulation are also observed with unfractionated heparin. For instance, Collen et al. (2000), using human microvascular endothelial cells on a fibrin-matrix model, observed that LMWH inhibits the stimulation induced by FGF-2, tumor necrosis factor-α, or vascular endothelial growth factor, whereas unfractionated heparin enhances it. In this study, we observed, using a Matrigel model, that unfractionated heparin had no effect on tubular morphogenesis, whereas LMWH enhanced it. Taking into account the major role of FGF-2 in tube formation, our findings may have implications in drug design.

The precise mechanism of action of polysaccharides on the cellular response to growth factors remains to be explored. Nevertheless, expression and/or activity of integrins seem to contribute to tubular morphogenesis of primary endothelial cells. Using flow cytometry, we unveiled overexpression of α6 in HUVEC cultivated in the presence of FGF-2 and LMWF (or LMWH). That α6 overexpression contributed to tubular morphogenesis and was not consecutive (or unrelated) to FGF-2-stimulation is supported by our observation that anti-α6 antibody abrogated tube formation. In contrast, we were unable to detect variation in the expression of the β1 subunit of the laminin receptor, even if, as for the anti-α6 antibody, the anti-β1antibody recognized the laminin receptor as well as the subunit by itself. Thus when HUVEC were treated with FGF-2, and LMWF (or LMWH), α6 expression increased, not that of β1: there was an imbalance in the ratio of the subunits on cell surface. Vascular tube formation results from a finely tuned balance between proliferation, migration and differentiation. In our study, LMWF and LMWH had no detectable effect (within 72 h) on HUVEC proliferation (data not shown). This observation is consistent with the study of Kroon et al. (1999) suggesting that tubular formation of endothelial cells is rather independent from their proliferation. Thus our data would be best understood by considering that α6 overexpression triggered differentiation of HUVEC but prevented proliferation. Such a picture is analogous to that anticipated by Sastry et al. (1999), using quail myoblasts, in which the α6 subunit regulates differentiation, whereas the β1 subunit triggers proliferation. More precisely, as long as the α6/β1 ratio remains less than unity, proliferation would occur; if the ratio increases, quail myoblasts no longer proliferate and start to initiate terminal differentiation. According to flow cytometry analysis, LMWF and LMWH enhanced FGF-2–induced α6 overexpression to a similar degree, yet only LMWF induced intense vascular tube formation. RT-PCR revealed that after LMWF stimulation, higher amounts of α6 mRNA were produced. Overall, our data suggest that α6 overexpression resulted from an up-regulation of transcription and that synthesis directly affected vascular tube formation. It is conceivable that higher levels of mRNA ultimately resulted in a higher amount of α6 subunit on HUVEC surface, when cells formed tubes on Matrigel (during the 18 h after seeding).

The importance of cell-surface heparan sulfate in the activation of signaling receptors by growth factors is attested to by studies providing evidence that their loss impedes binding of FGF-2 to FGFRs (Yayon et al., 1991). Binding of FGF-2 to FGFR seems to require heparan sulfate. To some extent, unfractionated heparin can substitute for the missing heparan sulfates in HSPG-deficient cells. The mechanism would involve a dimerization of FGF-2 that facilitates its interaction with FGFRs (Spivak-Kroizman et al., 1994). The same rationale would explain that, on the contrary, unfractionated heparin inhibits the binding of FGF-2 to FGFR on lymphoid cells bearing HSPG (Ornitz et al., 1992), simply because unfractionated heparin would compete with HSPG for the presentation of FGF-2 to FGFR. Consistent with this model, stripping off heparan sulfate from HUVEC with heparitinases resulted in loss of the FGF-2–induced α6 overexpres-

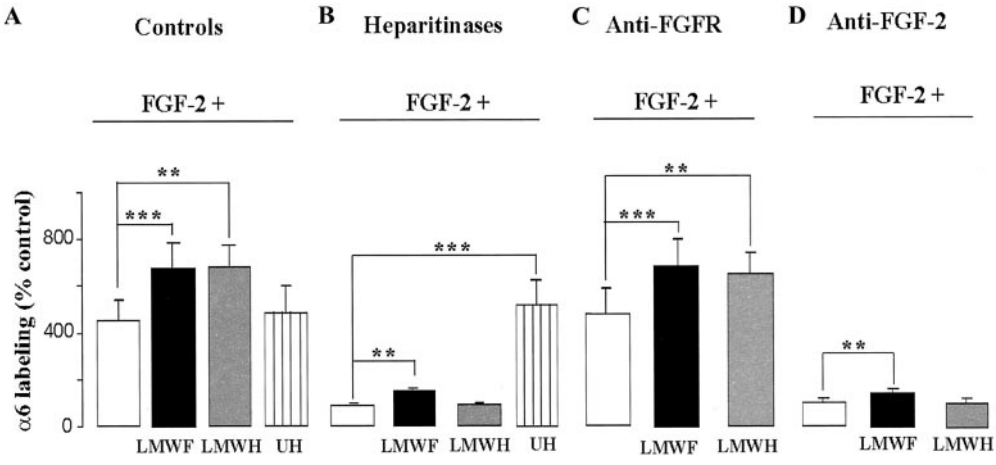

Fig. 3. Effect of HSPG, FGFR, and FGF-2 neutralization on the α6 surface labeling of HUVEC. Cells were grown for 72 h in medium supplemented with 5% FCS containing 5 ng/ml FGF-2 (□), FGF-2 and 10 μg/ml LMWF (■), FGF-2 and 10 μg/ml LMWH (▨), or FGF-2 and 10 μg/ml unfractionated heparin (▥). The specific contribution of HSPG was evaluated by a 1-h incubation at 37°C with 10 mU/ml of heparitinase I, II, and III before each feeding. The specific contribution of FGFR was evaluated by adding a blocking anti-FGFR antibody (10 μg/ml) during the 72-h culture. The specific contribution of FGF-2 was evaluated by adding a blocking anti-FGF-2 antibody (10 μg/ml) during the 72-h culture. Cell surface labeling of the α6 integrin subunit was quantified by flow cytometry. Data are expressed as percentage of the mean fluorescence intensity of untreated HUVEC (mean ± S.E. of three experiments). **, $p < 0.01$, ***, $p < 0.001$, significantly different from control.

sion. Adding unfractionated heparin partly restored stimulation, but neither LMWF nor LMWH restored stimulation to a similar extent. Undoubtedly, unfractionated heparin activated a different mechanism than LMWF (or LMWH). A simple explanation could be that LMWF and LMWH cannot present FGF-2 to FGFR (perhaps by lack of dimerization because of the shorter length of the polysaccharide chains). Unexpectedly, however, FGFR seemed dispensable for the FGF-2–induced α6 overexpression. Expression of α6 had an absolute requirement for heparan sulfates, but the mechanism of action seemed independent of FGFR. Thus, heparan sulfate would mediate directly the FGF-2–induced differentiation of HUVEC. Besides FGFR, heparan sulfates are known to mediate FGF-2 internalization in lymphoid cells (Rhogani and Moscatelli, 1992) and are directly implicated in FGF-2 signaling in myoblasts (Quarto and Amalric, 1994).

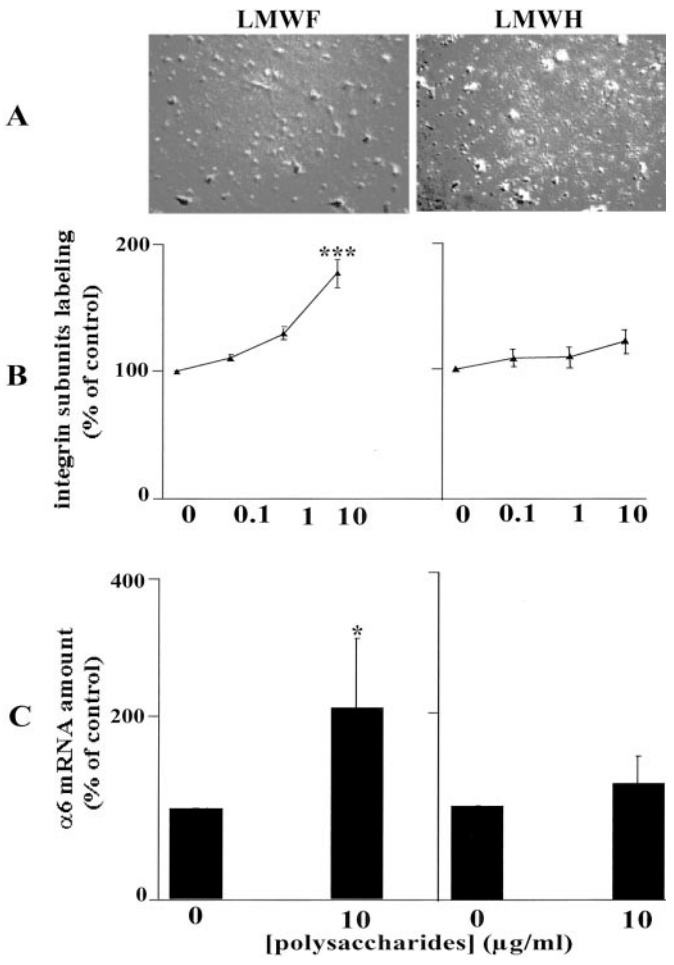

Fig. 4. LMWF triggers expression of α6 by itself. HUVEC were grown for 72 h in medium supplemented with 5% FCS containing either LMWF (left) or LMWH (right). A, cells incubated in the presence of 10 μg/ml polysaccharide were seeded onto Matrigel and analyzed by phase contrast microscopy 18 h later. Photographs are representative of a minimum of three experiments. B, cells incubated in the presence of variable amount polysaccharide (as indicated) were analyzed by flow cytometry for surface labeling of the α6 subunit. Data are expressed as percentage of the mean fluorescence intensity of untreated HUVEC (mean ± S.E. of seven experiments) as a function of the amount of polysaccharides added. C, cells incubated with or without 10 μg/ml polysaccharides (as indicated) were analyzed by real time RT-PCR for α6 mRNA transcripts. Data are expressed as percentage of the amount of α6 transcript compared with untreated cells (mean ± S.E. of three experiments in duplicate). *, $p < 0.05$, ***, $p < 0.001$, significantly different from control.

Overall, LMWF and LMWH may act as chaperones to shuttle FGF-2 to heparan sulfate, as has been suggested for other heparin-like molecules (Liekens et al., 1999; Thornton et al., 1999).

LMWF largely consists of fucose sulfate (Chevolot et al., 2001). In a recent study, we found that a fucosylated chondroitin sulfate extracted from sea cucumber potentiates FGF-2-induced tubular morphogenesis (Tapon-Bretaudière et al., 2002). Branched sulfated fucoses constitute the key motif in this activity, as demonstrated by a comparison of native and chemically modified fucosylated chondroitin sulfate. Thus, fucose seems critical for HUVEC differentiation induced by FGF-2. Many antibodies or carbohydrate-binding proteins, including selectins, bind to fucose-containing carbohydrates (Steegmaier et al., 1995; Boraston et al., 2003) Even in the absence of exogenous FGF-2, however, LMWF triggered a limited α6 overexpression in heparan sulfate-depleted HUVEC. Thus, another pathway for LMWF, different from those involving HSPG and/or FGFR, seemed to coexist in HUVEC. This pathway also triggered α6 expression. Despite a noticeable increase in α6, HUVEC stimulated with LMWF alone were unable to form tubes on Matrigel. Perhaps the amount of α6 remained insufficient to promote tubular morphogenesis. Expression of α6 would need to reach a threshold before initiating tube formation. Alternatively, in the absence of FGF-2, α6 subunits would remain in a conformation unsuitable for tube formation (Diamond and Springer, 1994; Hughes et al., 1996).

Overall, we demonstrated that LMWF enhanced FGF-2–induced tubular morphogenesis through α6 overexpression that was heparan sulfate-dependent. LMWF prevents arterial thrombus growth in animals with less hemorrhagic propensity than heparin (Millet et al., 1999; Colliec-Jouault et al., 2003) and inhibits smooth muscle cell proliferation (Logeart et al., 1997a,b). After myocardial infarction, endothelial cells increase expression of heparan sulfate proteoglycans, specifically at ischemic sites (Li et al., 1997; Kojima et al., 2001). LMWF, being antithrombotic and potentially proangiogenic, could constitute an interesting drug to initiate endothelial cell differentiation in revascularization of ischemic areas after myocardial infarction.

Acknowldgements. We are grateful to the nursing services of St. Vincent de Paul and Port Royal (Paris) for providing umbilical cords and to Prof. M. Aiach (INSERM U428) for helpful discussions.

References

Bauer J, Margolis M, Schreiner C, Edgell CJ, Azizkhan J, Lazarowski E, and Juliano RL (1992) In vitro model of angiogenesis using a human endothelium-derived permanent cell line: contributions of induced gene expression, G-proteins and integrins. *J Cell Physiol* **153:**437–449.

Boraston AB, Notenboom V, Warren RA, Kilburn DG, Rose DR, and Davies G (2003) Structure and ligand binding of carbohydrate-binding module CsCBM6-3 reveals similarities with fucose-specific lectins and "galactose-binding" domain. *J Mol Biol* **327:**659–669.

Carloni V, Romanelli RG, Mercurio AM, Pinzani M, Laffi G, Cotrozzi G, and Gentilini P (1998) Knockout of alpha6 beta1-integrin expression reverses the transformed phenotype of hepatocarcinoma cells. *Gastroenterology* **115:**433–442.

Chevolot L, Mulloy B, Ratiskol J, Foucault A, and Colliec-Jouault S (2001) A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae. *Carbohydr Res* **330:**529–535.

Collen A, Smorenburg SM, Peters E, Lupu F, Koolwijk P, Van Noorden C, and van Hinsbergh VW (2000) Unfractionated and low molecular weight heparin affect fibrin structure and angiogenesis in vitro. *Cancer Res* **60:**6196–6200.

Colliec-Jouault S, Millet J, Helley D, Sinquin C, and Fischer AM (2003) Effect of low-molecular-weight fucoidan on experimental arterial thrombosis in the rabbit and rat. *Thromb Haemostasis*, in press.

Diamond MS and Springer TA (1994) The dynamic regulation of integrin adhesiveness. *Curr Biol* **4:**506–517.

Fantl WJ, Johnson DE, and Williams MT (1993) Signalling by receptor tyrosine kinases. *Annu Rev Biochem* **62:**453–481.
Firsching A, Nickel P, Mora P, and Allolio B (1995) Antiproliferative and angiostatic activity of suramin analogues. *Cancer Res* **55:**4957–4961.
Giraux JL, Matou S, Bros A, Tapon-Bretaudière J, Letourneur D, and Fischer AM (1998) Modulation of human endothelial cell proliferation and migration by fucoidan and heparin. *Eur J Cell Biol* **77:**352–359.
Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ, and Ginsberg MH (1996) Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *J Biol Chem* **271:**6571–6574.
Klein S, Giancotti FG, Presta M, Albelda SM, Buck CA, and Rifkin DB (1993) Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol Biol Cell* **4:**973–982.
Kojima T, Takagi A, Maeda M, Segawa T, Shimizu A, Yamamoto K, Matsushita T, and Saito H (2001) Plasma levels of syndecan-4 (ryudocan) are elevated in patients with acute myocardial infarction. *Thromb Haemost* **85:**793–799.
Kroon ME, Koolwijk P, van Goor H, Weidle UH, Collen A, van der Pluijm G, and van Hinsbergh VW (1999) Role and localization of urokinase receptor in the formation of new microvascular structures in fibrin matrices. *Am J Pathol* **154:**1731–1742.
Li J, Brown LF, Laham RJ, Volk R, and Simons M (1997) Macrophage-dependent regulation of syndecan gene expression. *Circ Res* **81:**785–796.
Liekens S, Leali D, Neyts J, Esnouf R, Rusnati M, Dell'Era P, Maudgal PC, De Clercq E, and Presta M (1999) Modulation of fibroblast growth factor-2 receptor binding, signaling and mitogenic activity by heparin-mimicking polysulfonated compounds. *Mol Pharmacol* **56:**204–213.
Logeart D, Prigent-Richard S, Boisson-Vidal C, Chaubet F, Durand P, Jozefonvicz J and Letourneur D (1997a) Fucans, sulfated polysaccharides extracted from brown seaweeds, inhibit vascular smooth muscle cell proliferation. II. Degradation and molecular weight effect. *Eur J Cell Biol* **74:**385–390.
Logeart D, Prigent-Richard S, Jozefonvicz J, and Letourneur D (1997b) Fucans, sulfated polysaccharides extracted from brown seaweeds, inhibit vascular smooth muscle cell proliferation. I. Comparison with heparin for antiproliferative activity, binding and internalization. *Eur J Cell Biol* **74:**376–384.
Matou S, Helley D, Chabut D, Bros A, and Fischer AM (2002) Effect of fucoidan on fibroblast growth factor-2-induced angiogenesis in vitro. *Thromb Res* **106:**213–221.
Millet J, Colliec Jouault S, Mauray S, Theveniaux J, Sternberg C, Boisson Vidal C, and Fischer AM (1999) Antithrombotic and anticoagulant activities of a low molecular weight fucoidan by subcutaneous route. *Thromb Haemostasis* **81:**391–395.
Moscatelli D, Presta M, and Rifkin DB (1986) Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis and migration. *Proc Natl Acad Sci USA* **83:**2091–2095.
Murata J, Saiki I, Makabe T, Tsuta Y, Tokura S, and Azuma I (1991) Inhibition of tumor-induced angiogenesis by sulfated chitin derivatives. *Cancer Res* **51:**22–26.
Nardella A, Chaubet F, Boisson-Vidal C, Blondin C, Durand P and Jozefonvicz J (1996) Anticoagulant low molecular weight fucans produced by radical process and ion exchange chromatography of high molecular weight fucans extracted from the brown seaweed *Ascophyllum nodosum*. *Carbohydr Res* **289:**201–208.
Ornitz DM, Yayon A, Flanagan JG, Svahn CM, Levi E, and Leder P (1992) Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol Cell Biol* **12:**240–247.
Quarto N and Amalric F (1994) Heparan sulfate proteoglycans as transducers of FGF-2 signalling. *J Cell Sci* **107:**3201–3212.
Rabinovitz I, Nagle RB, and Cress AE (1995) Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype in vitro and in vivo. *Clin Exp Metastasis* **13:**481–491.
Rahmanian M, Pertoft H, Kanda S, Christofferson R, Claesson-Welsh L, and Heldin P (1997) Hyaluronan oligosaccharides induce tube formation of a brain endothelial cell line in vitro. *Exp Cell Res* **237:**223–230.
Rhogani M and Moscatelli D (1992) Basic fibroblast growth factor is internalized through both receptor-mediated and heparan sulfate-mediated mechanisms. *J Biol Chem* **267:**22156–22162.
Ribatti D, Urbinati C, Nico B, Rusnati M, Roncali L, and Presta M (1995) Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. *Dev Biol* **170:**39–49.
Rifkin DB and Moscatelli D (1989) Recent developments in the cell biology of basic fibroblast growth factor. *J Cell Biol* **109:**1–6.
Rosenberg RD, Shworak NW, Liu J, Schwartz JJ, and Zhang L (1997) Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? *J Clin Investig* **100:**67–75.
Saksela O, Moscatelli D, Sommer A, and Rifkin DB (1988) Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *J Cell Biol* **107:**743–751.
Sastry SK, Lakonishok M, Wu S, Truong TQ, Huttenlocher A, Turner CE, and Horwitz AF (1999) Quantitative changes in integrin and focal adhesion signaling regulate myoblast cell cycle withdrawal. *J Cell Biol* **144:**1295–1309.
Soeda S, Kozako T, Iwata K, and Shimeno H (2000) Oversulfated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: its possible mechanism of action. *Biochim Biophys Acta* **1497:**127–134.
Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D, Huang J, Jaye M, Crumley G, Schlessinger J, and Lax I (1994) Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation and cell proliferation. *Cell* **79:**1015–1024.
Steegmaier M, Levinovitz A, Isenmann S, Borges E, Lenter M, Kocher HP, Kleuser B, and Vestweber D (1995) The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor. *Nature (Lond)* **373:**615–620.
Tapon-Bretaudière J, Chabut D, Zierer M, Matou S, Helley D, Bros A, Mourao PA, and Fischer AM (2002) A fucosylated chondroitin sulfate from echinoderm modulates in vitro fibroblast growth factor 2-dependent angiogenesis. *Mol Cancer Res* **1:**96–102.
Thornton M, Barkley L, Mason JC, and Shaunak S (1999) Anti-Kaposi's sarcoma and antiangiogenic activities of sulfated dextrins. *Antimicrob Agents Chemother* **43:**2528–2533.
Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamijo T, Sugimoto T, Kaji K, Utsuyama M, and Kurashima C (1992) Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science (Wash DC)* **257:**1401–1403.
Yayon A, Klagsbrun M, Esko JD, Leder P, and Ornitz DM (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64:**841–848.
Yeh CH, Peng HC, and Huang TF (1999) Cytokines modulate integrin alpha(v-)beta(3)-mediated human endothelial cell adhesion and calcium signaling. *Exp Cell Res* **251:**57–66.
Zugmaier G, Lippman ME, and Wellstein A (1992) Inhibition by pentosan polysulfate (PPS) of heparin-binding growth factors released from tumor cells and blockage by PPS of tumor growth in animals. *J Natl Cancer Inst* **84:**1716–1724.

Address correspondence to: Dominique Helley, INSERM U428, Faculté des Sciences Pharmaceutiques et Biologiques Université Paris V, 4 avenue de l'Observatoire, 75270 Paris Cedex 06. E-mail: helley@pharmacie.univ-paris5.fr