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ACTIVITY OF PENTOSAN POLYSULPHATE AND DERIVED COMPOUNDS ON VASCULAR ENDOTHELIAL CELL PROLIFERATION AND MIGRATION INDUCED BY ACIDIC AND BASIC FGF *IN VITRO*

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Abstract—Pentosan polysulphate (PPS, SP 54, HEMOCLAR®), a highly sulphated semi-synthetic polysaccharide of MW 4700 Daltons is as efficient as heparin in potentiating the mitogenic activity of acidic FGF (aFGF) on human umbilical vein endothelial cells (HUVEC). When added to basic FGF (bFGF), no effect was observed on these cells. However, PPS had a strong inhibitory effect on the growth of bovine aortic endothelial cells (BAEC), as did heparin. PPS was fractionated according to molecular weight and the activities of these fractions were compared. A PPS fraction of MW = 3200 Daltons represented the critical size required to affect cell proliferation induced by FGFs. We also report that acidic and basic FGFs are both chemotactic for BAEC and HUVEC. PPS and heparin, which were chemotactic alone on BAEC, potentiated acidic FGF-induced migration but inhibited the chemotactic response of basic FGF. These data suggest that PPS, although having a different structure, can mimic the *in vitro* activity of heparin on FGF-induced proliferation and migration of endothelial cells and thus the possibility of a specific heparin sequence being involved in the interactions with FGFs can be questioned.

The availability of cultured endothelial cells constituted a useful tool for studying endothelium repair in wound healing and atherosclerosis and also capillary endothelial cell proliferation and migration in angiogenesis. Among the factors to be described as determining in these processes were acidic and basic FGFs† (aFGF and bFGF) [1], two closely related peptides with high affinity for heparin [2]. They have been purified to homogeneity using heparin Sepharose affinity chromatography from various tissues including brain, hypothalamus, retina, placenta, adrenal gland and various tumors [3, 4]. *In vitro*, both growth factors stimulated the proliferation of multiple endothelial cell types isolated from arteries or veins [5, 6], or capillaries [7]. They also induced a chemotactic response of these cells [8–10]. *In vivo*, these heparin binding growth factors induced angiogenesis in nanogram amounts in the chick embryo chorioallantoic membrane (CAM) and in the rabbit cornea [2, 11, 12]. Angiogenic substances like FGFs are modulated by heparin. *In vivo*, heparin augmented angiogenesis induced by aFGF or by a tumour implanted in the CAM [2, 13], but also inhibited angiogenesis when coadministered with cortisone or hydrocortisone [14–16]. *In vitro*, heparin stimulated locomotion of capillary endothelial cells and potentiated the effects of aFGF [8, 17]. Heparin has been shown to protect aFGF from inactivation [18], explaining its potentiating effect for aFGF proliferative activity on human endothelial cells [7, 19]. However, heparin was a potent inhibitor of capillary endothelial cell growth regardless of whether cells were exposed to basic or acidic FGF [7, 20], suggesting a strict dependence on cell type for the effects of heparin on cultured vascular endothelial cell proliferation. These *in vitro* observations raised a discrepancy with the *in vivo* results, where heparin potentiated capillary growth in combination with aFGF in the CAM assay. The reasons for such differences still remain unclear. These observations raised the question of the selectivity and the specificity of heparin effects on FGFs. Indeed, one could imagine that the heparin affinity observed could be attributed to a specific heparin fragment with high affinity for FGFs as already demonstrated for heparin binding on antithrombin III [21]. To our knowledge, the only structure/activity relationship study which has been made on this subject demonstrated that a synthetic pentasaccharide with high affinity for antithrombin III could also enhance the mitogenic properties of aFGF on non-vascular cells (hamster fibroblasts or bovine epithelial lens cells) [22]. Furthermore, other sulphated polysaccharides, such as dextran sulphates, were claimed to be potent inducers of aFGF mitogenicity for cultured human endothelial cells [23].

In this paper, we have compared the effects of pentosan polysulphate (PPS, SP 54, HEMOCLAR®) and heparin on human umbilical vein and bovine aorta endothelial cell proliferation and migration.

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† Abbreviations used: BAEC, bovine aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; PPS, pentosan polysulphate; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; MW, molecular weight; FCS, fetal calf serum; NCS, newborn calf serum.

PPS is obtained from beechwood, has a relatively low molecular weight (MW = 4700 Daltons), is highly sulphated (1.9 sulphate group per saccharide unit) and consists of a β $(1 \rightarrow 4)$ chain of D-xylose units with a α $(1 \rightarrow 2)$ linkage to every ninth xylose unit on average [24]. PPS is thus very different from heparin in many points (molecular weight, sugar composition, sulphation degree, etc.). It has been recently described as a potent inhibitor of vascular smooth muscle cell growth *in vitro* [25].

MATERIALS AND METHODS

Chemicals. Heparin (sodium salt, from pig intestinal mucosa), fibronectin (from bovine plasma) and collagenase (type II) were purchased from Sigma. Medium 199, Dulbecco's modified Eagle medium (DMEM), fetal and newborn calf sera (FCS and NCS), glutamin, penicillin and streptomycin sulphate were from Boehringer Mannheim (France). Pentosan polysulphate (PPS) was from Sanofi Recherche. Fractionation of PPS was performed by ultrafiltration on Amicon hollow fibre cartridges (fractions 1–5). The highest molecular weight fraction (F6) was prepared from artificially sulphated hemicellulose according to Whistler and Feather [26]. We may assume that such a high molecular weight population is present in low proportion within the unfractionated PPS. Indeed, this material was indistinguishable, in terms of uronic acid content (1 per 9 oxylase) and sulphation (1.94 per saccharide), from the fraction with the same MW, purified by gel fractionation of a large quantity of PPS. All other chemicals were from Prolabo, France.

FGF purification. Bovine pituitary-derived basic FGF was purified using heparin sepharose affinity chromatography as previously described [27]. Bovine brain-derived acidic FGF was purified using a similar procedure [28]. All samples were kept in frozen aliquots at −80° in DMEM + 0.5% bovine serum albumine (BSA) after assessment of their homogeneity by reverse phase HPLC using an ALTEX ultrapore C3 column (4.6 × 75 mm, Beckman, Berkeley, CA).

Furthermore, analytical SDS-PAGE was performed on gradient gels (8–25%) in presence of dithiothreitol according to Gospodarowicz *et al.* [27]. Gels were first silver-stained and then integrated with a laser densitometer (LKB). By these two methods, homogeneity of the samples was assumed to be greater than 95% for both FGFs.

Cell culture. Endothelial cells from umbilical vein (HUVEC) were obtained by collagenase dissociation according to Jaffe *et al.* [29]. Cells were grown in the presence of medium 199 supplemented with 10% FCS and 10% NCS, 4 mM glutamin, 50 U/ml of penicillin and 50 μg/ml of streptomycin sulphate. For experiments, cells were used in the first or the second passage. Bovine aortic endothelial cells (BAEC) were obtained from aortas of adult cows by scraping as previously described [30]. BAEC were routinely cultured in DMEM supplemented with 10% calf serum, 50 U/ml of penicillin and 50 μg/ml of streptomycin sulphate. Bovine basic FGF (1 ng/ml) was added every other day. Those two cell types were identified as vascular endothelial cells on the basis of positive staining for factor VIII R:Ag as determined by direct immunofluorescence [31].

Cell growth measurement. For measurement of cell growth, bovine aorta or human umbilical vein endothelial cells were respectively seeded in DMEM + 10% NCS or medium 199 + 10% FCS + 10% NCS at 2×10^4 cells per well (24 wells plate, Nunclon, Denmark), in the presence of various concentrations of growth factors. The tested compounds were added simultaneously at different concentrations to replicate sparse cultures of endothelial cells in culture medium. Medium was replaced after 2 days in culture. BAEC were cultured in plastic dishes and HUVEC were grown on fibronectin coated plates (coating with 0.1 mg/ml of fibronectin for 24 hr at 4°). For growth rate determinations, after 4 days in culture, triplicate plates were trypsinised and the cells counted with a Coulter counter (Coultronics).

Chemotaxis assay. Chemotaxis assays were carried out using a 48 microwell apparatus (Neuroprobe, Cabin John, MD) as described [32, 33]. Test solutions in DMEM + 0.2% BSA or control media were loaded into each bottom well. A nucleopore membrane with 8 μm pores from Neuroprobe (Cabin John, MD) was applied to the plate. The top plate with upper wells was attached and the apparatus was equilibrated at 37° for 15 min. The cells used in the assay were detached from the culture flask by trypsin treatment (0.05% trypsin, 0.02% EDTA). After centrifugation (400 *g*, 10 min), the cells were resuspended in DMEM + 10% FCS (final concentration: 40×10^4 cells/ml). Cell suspensions were loaded into each top chamber of the microwell plate (20×10^3 cells/well) and the apparatus was incubated at 37° with 5% CO_2 for 5 hr. After disassembling, the non-migrated layer of cells was removed from the membrane using a rubber wiper blade (Neuroprobe). The migrated cells were fixed to the membrane and stained using a M + D Diff quick staining set (Dade, France). The membrane was rinsed with H_2O and the cells which had migrated were counted under a microscope (250× magnification). Five fields were counted for each well and 4 replicate wells were run for each experimental protocol. The number of cells per field was converted to cells per mm^2 (0.18 mm^2/field) and averaged. Results were expressed as a percentage of migrating cells per total number of cells originally placed in each well.

RESULTS

Effects of bovine acidic and basic FGF on the proliferation of BAEC and HUVEC in culture

The effect of basic and acidic FGF on the proliferation of low density BAEC and HUVEC is shown in Fig. 1. The addition of bFGF greatly improved the growth rate of both cell types. The concentration of bFGF required to get optimal cell proliferation (A_{max}) was 1 ng/ml in both cases. Acidic FGF, when tested at concentrations ranging from 0.1 to 100 ng/ml, had a less marked effect on cell growth. The A_{max} as well as the concentration of aFGF required to give half maximum response (ED_{50}), which was between 10 and 20 ng/ml, was 30–

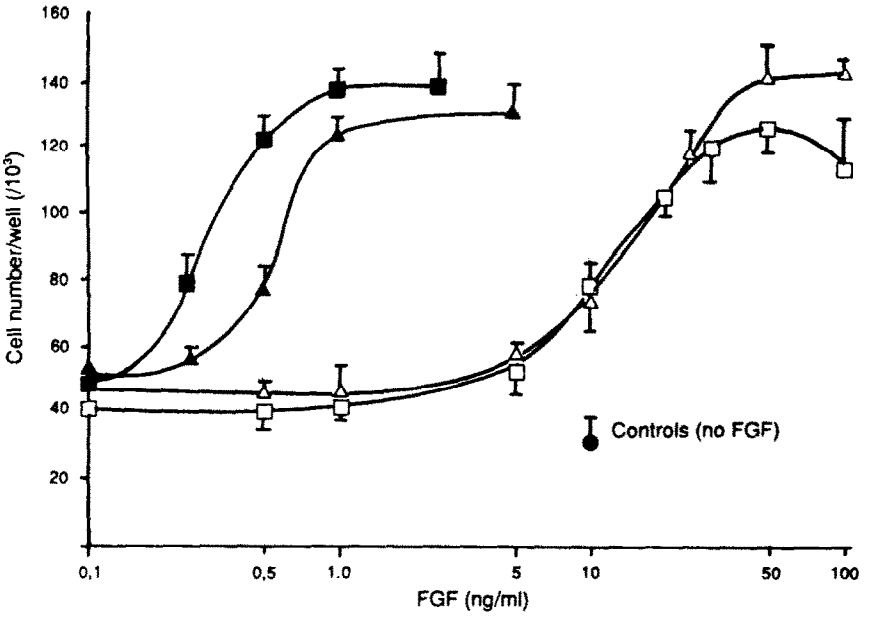

Fig. 1. Effect of basic and acidic FGF on the proliferation of BAEC and HUVEC. Sparse cultures of BAEC (squares) and HUVEC (triangles) (20×10^3 cells/well) were respectively grown in DMEM + 10% FCS or Medium 199 + 10% FCS + 10% NCS with the indicated concentrations of bFGF (full symbols) or aFGF (empty symbols). After 4 days in culture, cells were trypsinized and counted. Data are reported as mean cell density ± SD for 3 replicate cultures.

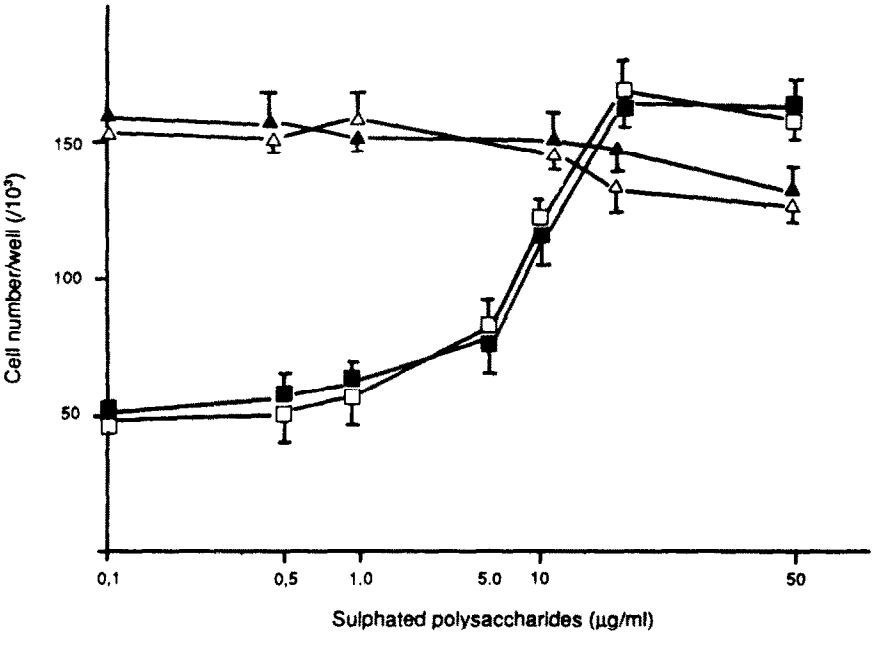

Fig. 2. Effect of PPS and heparin on the proliferation of HUVEC exposed to bovine acidic FGF (1 ng/ml) and basic FGF (1 ng/ml). Low density HUVEC cultures (20×10^3 cells/well) were seeded on fibronectin coated wells and exposed to Medium 199 + 10% FCS + 10% NCS supplemented with a fixed concentration (1 ng/ml) of bFGF (triangles) or aFGF (squares). Increasing concentrations of PPS (full symbols) or heparin (empty symbols) were added simultaneously. After 4 days in culture, triplicate wells were trypsinized and cells were counted. Data are reported as mean cell density ± SD.

40-fold higher than those observed for bFGF. These observations are consistent with already published results [7].

Effects of pentosan polysulphate and heparin on the proliferation of HUVEC and BAEC exposed to acidic or basic FGF

When different concentrations of standard heparin were added in combination with aFGF to sparse cultures of HUVEC, we observed a drastic increase in cellular proliferation. These results are identical to those already described by others [7, 19] (Fig. 2). Pentosan polysulphate (PPS) was tested at the same concentrations. It was able to drastically increase the mitogenic activity of aFGF (1 ng/ml) from 5 μg/ml with maximum activity at 10 μg/ml. At this concentration, PPS restored the ability of aFGF to stimulate the proliferation of HUVEC to the same level as bFGF. When added in combination with bFGF (1 ng/ml), neither heparin nor PPS modified the potency of bFGF. Heparin or PPS alone did not show any marked effect on HUVEC growth (not shown). In contrast, when incubating BAEC with PPS or heparin in the presence of acidic or basic FGF under the experimental conditions exposed above, a strong antiproliferative effect was observed (Fig. 3). Standard heparin, at concentrations as low as 0.1 μg/ml, significantly reduced the proliferative response to optimal concentrations of aFGF (50 ng/ml) and bFGF (1 ng/ml) (IC_{50}s were 150 ng/ml and 22 ng/ml respectively). PPS similarly inhibited the growth of BAEC stimulated by acidic or basic FGF (IC_{50} = 800 ng/ml and 260 ng/ml respectively). In order to document adequately these effects of sulphated polysaccharides on BAEC proliferation, we used a colorimetric assay developed by Mosmann [35]. The assay is dependent on cellular reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by the mitochondrial succinate-deshydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. With the highest concentration of sulphated polysaccharide we used (10 μg/ml), PPS or heparin in addition to acidic or basic FGF (50 ng/ml and 1 ng/ml respectively) did not show any detectable difference in MTT reduction compared with the same number of untreated cells (not shown). These results indicated that no cytotoxicity of the tested compounds was observed.

Effect of PPS fractions on the proliferation of HUVEC and BAEC induced by acidic or basic FGF

In order to define more precisely the minimal size required to potentiate or reduce (depending on cell type) the mitogenic activity of acidic or basic FGF, fractions of PPS were prepared according to molecular size (see Methods). These fractions showed comparable sulphatation degrees (1.9 sulphate group per saccharide unit) and uronic acid content. All fractions were tested for their action on cell growth at the same concentration: 10 μg/ml (A_{max} of PPS) when co-incubated with acidic or basic FGF on HUVEC; 0.5 μg/ml (IC_{50} of PPS) when added to the same growth factors on BAEC. When PPS fractions were incubated with a FGF on HUVEC, all fractions but F1 potentiated the mitogenic activity of the

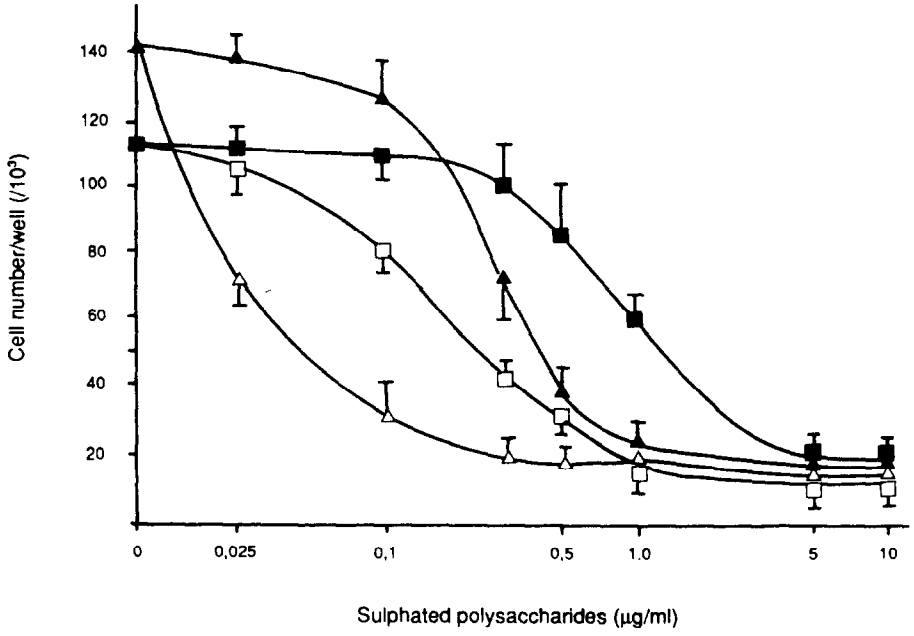

Fig. 3. Effect of PPS and heparin on the growth of BAEC induced by acidic FGF (50 ng/ml) or basic FGF (1 ng/ml). Sparse cultures of BAEC (20×10^3 cells/well) were allowed to grow in the presence of DMEM + 10% FCS supplemented with aFGF (50 ng/ml) (squares) or bFGF (1 ng/ml) (triangles). Increasing concentrations of PPS (full symbols) or standard heparin (empty symbols) were added simultaneously. After 4 days in culture, triplicate wells were trypsinized and cells were counted. Data are expressed as mean cell density ± SD.

Table 1. Molecular weight dependence for PPS fractions on the growth of HUVEC and BAEC stimulated by acidic or basic FGF

PPS fractions	MW (kDa)	% increase in HUVEC growth		% inhibition of BAEC proliferation	
		aFGF	bFGF	aFGF	bFGF
PPS	4.7	320	3	22	74
F1	2.2	8	−4	7	14
F2	3.2	288	7	21	53
F3	5.3	317	9	47	73
F4	6.7	301	9	61	81
F5	9.6	328	−2	79	89
F6	40	340	17	97	98

Compounds were added at 10 μg/ml or 0.5 μg/ml to replicate sparse cultures (20×10^3 cells/well) of HUVEC or BAEC respectively, in medium supplemented with aFGF (1 ng/ml on HUVEC, 50 ng/ml on BAEC) or bFGF (1 ng/ml), and the cells were counted 4 days later. Potentiation or inhibition of cell growth was calculated with reference to growth in cultures containing the FGFs alone.

growth factor to the same level as already observed for PPS (Table 1). On the other hand, these compounds did not present any effect on the mitogenic potency of bFGF, on the same cells. In contrast, fractionation of PPS by molecular size showed that inhibition of BAEC proliferation stimulated by acidic or basic FGF was directly related to molecular weight. The lowest molecular weight fraction (2.2 kDa) had no marked effect on cell growth while the largest fraction (F6) presented a strong inhibitory effect at 0.5 μg/ml with both FGFs. None of the compounds tested had any effect on cell viability as assessed by trypan blue exclusion.

Effect of acidic and basic FGF on BAEC and HUVEC migration

Many factors stimulating proliferation also stimulate migration of vascular endothelial cells [8, 17, 34]. When HUVEC were placed in the upper compartment of a modified Boyden chamber, we observed that increased migration occurred with as little aFGF as 10 ng/ml in the lower compartment (Fig. 4). For bFGF, significant migration was observed for the same concentrations. In both cases, the optimum concentration which gave maximum migration was 500 ng/ml. Basic FGF was 2–3-fold more potent than aFGF in inducing chemotaxis of

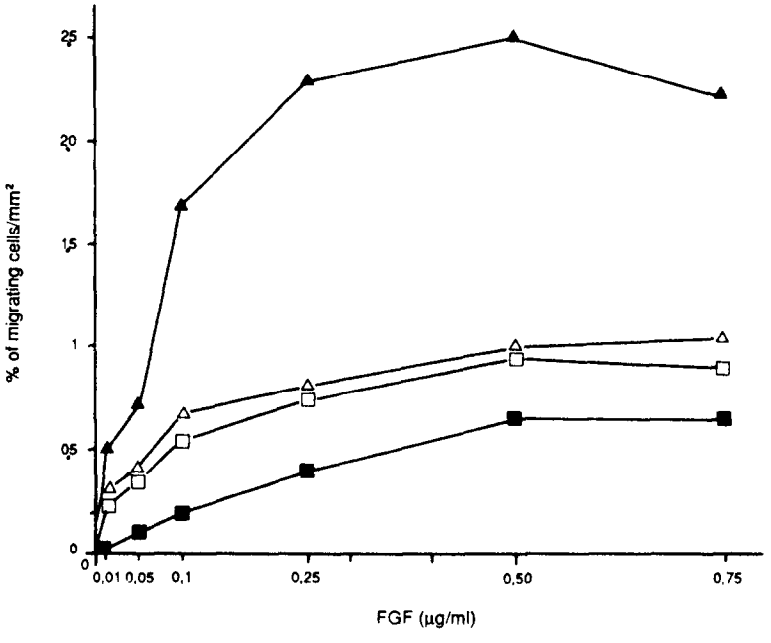

Fig. 4. Chemotaxis effect of acidic and basic FGF on BAEC and HUVEC. Increasing concentrations of aFGF (empty symbols) of bFGF (full symbols) were analysed for their ability to promote chemotaxis in the Boyden chamber on BAEC (squares) or HUVEC (triangles) (for experimental details see Materials and Methods). Results are expressed as per cent of migrating cells per total number of cells added in each well (20×10^3 cells/well). Data are reported as mean cell number for 3 replicate chemotaxis assays.

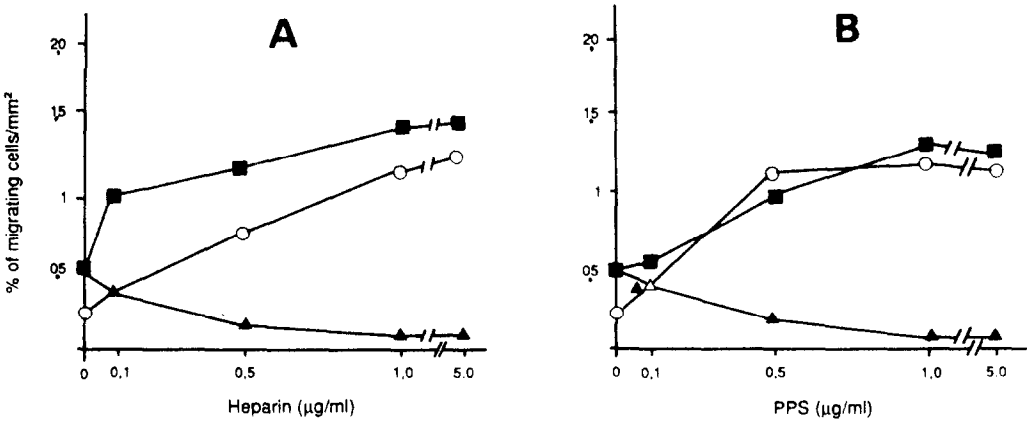

Fig. 5. Chemotactic response of BAEC to PPS or heparin alone or coincubated with acidic or basic FGF. Chemotaxis was assayed in the modified Boyden chamber (for experimental details see Materials and Methods). (A) Heparin was added alone (○) or in combination with aFGF (100 ng/ml) (■) or bFGF (340 ng/ml) (●) at the indicated concentrations. (B) PPS was added alone (○) or in combination with aFGF (100 ng/ml) (■) or bFGF (340 ng/ml) (●) at the indicated concentrations. Data are expressed as per cent of migrating cells per total number of cells added in each well (20×10^3 cells/well). Results are reported as mean cell number for 3 replicate chemotaxis assays.

HUVEC. On BAEC, we observed that acidic and basic FGF stimulated endothelial cell migration within the same range of concentrations (0.01–0.75 ng/ml). Although aFGF stimulated BAEC migration at the same level as already observed for HUVEC, this response represented for BAEC, only 30–40% of the chemotactic response observed for bFGF on HUVEC. The optimal concentration of acidic and basic FGF for maximum chemotaxis on this cell type, as already observed for HUVEC, was 500 ng/ml.

Effect of PPS and heparin on acidic and basic FGF-induced chemotaxis on BAEC

In these experiments, standard heparin was used alone or in combination with aFGF (100 ng/ml) or bFGF (340 ng/ml). Figure 5A indicates a significant chemoattractant effect of heparin from the concentration of 0.5 μg/ml which reached a maximum at 1 μg/ml. The addition of both aFGF and heparin significantly increased the chemotactic response of the cells relative to the heparin response. This effect occurred with as low as 0.1 μg/ml of heparin. However, heparin in combination with bFGF showed marked inhibition of the chemotactic response of the cells to bFGF ($IC_{50} = 0.3$ μg/ml). Similar results were obtained with PPS alone or in combination with bFGF, indicating that the same mechanisms were involved in this process (Fig. 5B). However, when PPS was co-incubated with aFGF, no significant potentiating effect was observed for this compound.

DISCUSSION

In the present paper, we first confirmed previous reports establishing that heparin can potentiate the mitogenic activity of aFGF on HUVEC *in vitro* [7, 19, 23]; in these conditions, aFGF became as potent as bFGF in stimulating HUVEC growth. This action of heparin was explained by a protective effect of aFGF from inactivation [18]. We further showed that PPS, a semi-synthetic sulphated polysaccharide, structurally very different from heparin, can similarly greatly potentiate, in a dose-dependent manner, the mitogenic activity of aFGF on HUVEC proliferation. Fractionation of PPS according to molecular size revealed that smaller fragments (tetrasaccharides) had lost virtually all potentiating activity of aFGF. From these experiments, we found that the effect of PPS was unrelated to the mean molecular weight, over a range of 3.2 to 40 kDa, suggesting that the minimal PPS fragment required to potentiate aFGF mitogenicity could have the size of a hexasaccharide. As observed for heparin or unfractionated PPS, none of the compounds tested had any effect on bFGF-induced mitogenesis. In contrast, on BAEC, PPS and heparin strongly inhibited either acidic or basic FGFs-induced growth. On these cells, molecular weight of PPS fractions was a critical determinant for the inhibition of the mitogenic potencies of both FGFs. This inhibitory activity could therefore be attributed to a different mechanism from the one already observed in the interactions of PPS with aFGF or bFGF on HUVEC. Studies are currently under investigation to define more precisely whether PPS or its related fractions could act as potent antagonists either on FGF itself and/or on its binding sites on bovine aorta endothelial cells or affect the DNA synthesis. It is of interest to note that the strong inhibitory effect of PPS or heparin on BAEC growth reflects almost the same situation as already encountered for heparin on bovine capillary endothelial cells [7]. Indeed, Gospodarowicz *et al.* [7] observed that a marked antiproliferative activity was obtained for both FGFs when heparin was added within the same range of concentrations we used. These *in vitro* effects question the relevance of such observations, considering that heparin markedly enhances the angiogenic activity of aFGF *in vivo* [2, 11, 12].

In another part of this paper, we emphasized the interactions of sulphated polysaccharides like heparin or PPS with acidic or basic FGFs on another aspect of the endothelium repair process: the migration of endothelial cells. This event is crucial in angiogenesis. Both acidic and basic FGFs stimulated the motility of BAEC and HUVEC. Heparin was also chemotactic for BAEC and was shown to potentiate chemotaxis of aFGF. Surprisingly, our results raised discrepancies with the observations of Azizkhan *et al.* [17], who demonstrated that heparin did not stimulate by itself migration of endothelium from bovine aorta to such an extent. However, this might reflect differences in experimental conditions. In the same experiment, heparin inhibited the chemotactic activity of bFGF. PPS, although it did not potentiate the chemotactic activity of a FGF as heparin did, increased on its own the migration of BAEC in the modified Boyden chamber assay. PPS inhibited bFGF-induced BAEC motility to the same extent as heparin.

In conclusion, we have shown that PPS, a sulphated polysaccharide structurally different from heparin, presented *in vitro* almost the same effects as heparin on the proliferation and the migration of HUVEC and BAEC induced by acidic or basic FGFs. One could therefore suspect that FGF molecules, which tightly bind heparin as reflected by the extremely selective method of heparin sepharose affinity chromatography used for their purification [27, 28], would also bind sulphated polysaccharides from other origins. Thus, our results question a possible affinity sequence for acidic or basic FGFs in the heparin chains, as already observed for other proteins (like antithrombin III) interacting with heparin [21].

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