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HEPARIN-LIKE GLYCOSAMINOGLYCANS INFLUENCE GROWTH AND PHENOTYPE OF HUMAN ARTERIAL SMOOTH MUSCLE CELLS IN VITRO. II. THE PLATELET-DERIVED GROWTH FACTOR A-CHAIN CONTAINS A SEQUENCE THAT SPECIFICALLY BINDS HEPARIN

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SUMMARY

Synthetic oligopeptides were used to study the specificity of the interaction between heparin and platelet-derived growth factor (PDGF) in competition experiments. DNA synthesis in PDGF-dependent human arterial smooth muscle cell (hASMC) cultures was used as a biological tracer of PDGF activity. Oligo-108-124 (corresponding to amino acid residues 108-124 of the long PDGF A-chain isoform) had no effect on DNA synthesis in itself but competed at 10^{-10} *M* concentration effectively with PDGF for binding to heparin and released the block on thymidine incorporation induced by heparin. Poly-lysine-serine (lysine:serine ratio 3:1) was also effective but at a considerably higher concentration (10^{-6} *M*). Poly-arginine-serine did not compete with PDGF for heparin as deduced from the cell assay. This suggested that among basic amino acids, lysine was more important than arginine for heparin binding. Deletion of lysine residues 115 and 116 in Oligo-108-124 abolished its effect on the interaction between PDGF and heparin in the cell assay. Likewise, Oligo-69-84 (corresponding to the PDGF A-chain residues 69-84), with three lysine residues interrupted by a proline, was ineffective. In Oligo-108-124, the lysine residues are interrupted by an arginine. Our results suggested that the binding between PDGF and heparin is specific and that the amino acid sequence [-Lys^{115}-Lys^{116}-Arg^{117}- Lys^{118}-Arg^{119}-] is of major importance. They do not however, exclude other domains of the PDGF A or B chains as additional binding sites for heparin nor do they exclude the possibility that heparin and the PDGF receptor share a common binding site.

Key words: heparin; PDGF; smooth muscle cells; human; atherosclerosis.

INTRODUCTION

The platelet-derived growth factor (PDGF) has been associated with arterial smooth muscle cell (ASMC) migration (Grotendorst et al., 1981, 1982) and proliferation (Ross, 1981; Clemmons, 1984; Seifert et al., 1984; Owens et al., 1986; Fager et al., 1988) in vitro. In the presence of mitogens, heparin and heparin-like glycosaminoglycans (GAGs) inhibit ASMC proliferation in vivo (Clowes and Karnovsky, 1977) as well as in vitro (Hoover et al., 1980; Castellot et al., 1981, 1982; Nilsson et al., 1983; Majack and Bornstein, 1984; Fritze et al., 1985; Majack et al., 1985; Fager et al., 1988, 1989). Heparin-like GAGs have also been shown to inhibit ASMC migration in vitro (Castellot et al., 1981; Majack and Clowes, 1984). Heparin-like GAGs constitute a small but significant proportion of the GAGs in proteoglycans of the atherosclerotic arterial wall (Dalferes et al., 1987). Furthermore, heparin-like GAGs are produced in vitro by vascular endothelial (Castellot et al., 1981, 1982) and smooth muscle (Fritze et al., 1985) cells.

We have previously found that human ASMCs (hASMCs) depended on exogenous PDGF for their proliferation in vitro (Fager et al., 1988). Our hASMC did not express mRNA for the A and B chains of PDGF. In plasma-derived serum or in 1% bovine serum albumin (BSA), hASMCs became reversibly growth arrested and differentiated to express smooth muscle specific contractile proteins in stress fiber structures (Fager et al., 1989). The mitogenic activity of serum was effectively counteracted by anti-PDGF IgG or by heparin in the medium. The growth inhibition and differentiation observed in cultures in medium containing heparin and serum was directly related to the ratio heparin:serum and was fully reversible when heparin was withdrawn from the medium (Fager et al., 1988).

When hASMCs were cultured in the presence of heparin and serum, PDGF beta receptors were expressed on the cell surface as judged from indirect immunofluorescence (Fager et al., 1988). This was in contrast to the down-regulation observed in the absence of heparin, suggesting that the growth inhibitory effect of heparin might be due to interference with the binding of PDGF to its cell surface receptor.

In the companion article (Fager et al., 1991) we found that this was indeed the case. In 0.15 *M* NaCl, Heparin-Sepharose CL 6B bound serum mitogen(s). The unbound serum fraction was non-mitogenic when added to hASMCs in serum-free medium. Its mitogenic effect was, however, restored by the addition of PDGF dimers (AA, AB, or BB) or of a fraction (RF I) that dissociated from Heparin-Sepharose at about 0.4 *M* NaCl. Anti-PDGF IgG as well as heparin blocked the mitogenic effect of these combinations on hASMCs. We could, however, not disclose whether heparin and the PDGF receptor bound to the same domain of PDGF nor if the

binding of PDGF to heparin was only an unspecific ionic interaction.

We have, therefore, undertaken to study the competition between PDGF and synthetic peptide fragments for heparin to elucidate the specificity of the interaction with mitogenicity for hASMCs as a biological endpoint.

Materials and Methods

Human serum, growth factors, chemicals and culture materials. Human serum and low density lipoproteins (LDL) were isolated as previously described (Fager et al., 1991). The retained fractions RF I (containing PDGF) and RF II [possibly containing fibroblast growth factor (FGFs)] as well as the unretained fraction UF (devoid of these mitogens) from affinity chromatography of human serum on Heparin-Sepharose CL 6B were prepared as before (Fager et al., 1991).

Recombinant PDGF AA (rPDGF-AA) and BB (rPDGF-BB) have been characterized (Östman et al., 1989) and were provided by Dr. Carl-Henrik Heldin, (Uppsala, Sweden). Heparin sodium pure (powder) from porcine intestinal mucosa was a gift from Leo Pharmaceutical Products (Ballerup, Denmark). Bovine serum albumin (BSA) (Fraction V, Cat. no. A 4503), Poly(Lys HBr,Ser) with a lysine:serine ratio of 3:1 (Poly-Lys-Ser) [molecular weight (MW) 31 000, Cat. no. P-9160], Poly-L-Lysine (Poly-Lys) (MW 3300, Cat. no. P-0879) and ovalbumin from chicken egg (grade V, MW 42 000, Cat. no A 5503) were from Sigma Chemical Co. (St. Louis, MO). Human fibronectin (Cat. no 40008) was from Collaborative Research Inc. (Bedford, MA) and Heparin-Sepharose CL 6B from Pharmacia (Uppsala, Sweden). ^{125}I-Labeled recombinant PDGF(c-sis) [rPDGF(c-sis)] (Cat. no. IM 213) and [6-^{3}H]thymidine (Cat. no. TRK 61) were from Amersham (Buckinghamshire, UK). Cell culture media, trypsin, fetal bovine serum, and culture vessels were from Flow Laboratories (Irvine, Scotland) and Lab-Tec tissue culture eight chambers/slide from Miles Laboratories (Naperville, IL).

Peptide synthesis. Oligopeptides were synthesized in a MilliGen 9050 PepSynthesizer utilizing 9-fluorenylmethyloxycarbonyl to provide temporary alpha-amino protection (Fmoc-polyamide method) as recommended by the manufacturer (Millipore, Bedford, MA). The crude peptides were precipitated in diethyl ether and the dried pellets dissolved in water. The peptides were then purified by preparative high performance liquid chromatography (HPLC) on a LiChrosorb RP-18 (7 μm) column (E. Merck, Darmstadt, Germany) utilizing an LKB 2152 gradient former and an 2150 HPLC pump (LKB, Stockholm, Sweden). Peptides were eluted in a gradient (0 to 80%, vol/vol) of acetonitrile in water containing 0.1% (vol/vol) trifluoroacetic acid. Purified peptides were lyophilized and stored at −20° C.

One oligopeptide (Oligo-69-84) corresponded to amino acid residues 69–84 (NH_2-Val-Glu-Tyr-Val-Arg-Lys-Lys-Pro-Lys-Leu-Lys-Glu-Val-Gln-Val-Arg-COOH) (MW 1999) of the PDGF A-chain isoforms. Another (Oligo-108-124) corresponded to amino acids 108–124 (NH_2-Gly-Arg-Pro-Arg-Glu-Ser-Gly-Lys-Lys-Arg-Lys-Arg-Lys-Arg-Leu-Lys-Pro-COOH) (MW 2180) of the alternatively spliced long A-chain isoform. Two oligopeptides were prepared as controls. One peptide, Oligo-(115+116-del) (MW 1922), corresponded to Oligo-108–124 except for a deletion of lysine residues 115 and 116. The other control peptide was Oligo-Arg-Ser (NH_2-Arg-Ser-Arg-Ser-Arg-Ser-Arg-Ser-Arg-COOH) (MW 1150). LDL, fibronectin, and ovalbumin were also used as control peptides.

The quality of purified synthetic oligopeptides was controlled by analytical HPLC utilizing the LKB system and a LiChrosphere 100 RP (18.5 μm) column (E.Merck). The amino acid sequence was controlled by sequencing 1 n*M* of peptide in 80% acetonitrile (vol/vol) in a 477A Protein Sequencer with a PTH 120A detection unit (269 nm) (Applied Biosystems, Foster City, CA).

Circular dichroism (CD) spectra of synthetic peptides were recorded at room temperature using a JASCO J-500A recording spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) with 1-mm path length (Greenfield and Fasman, 1969). The CD measurements were performed in phosphate buffered saline (PBS) and corrected for buffer absorption. First, the CD spectrum of the peptide (0.1 m*M*) in PBS was recorded and then the spectrum of the peptide (0.1 m*M*) together with heparin (1 μ*M*) in PBS.

Heparin affinity chromatography. The binding of test substances to heparin was first evaluated by affinity chromatography on Heparin-Sepharose CL 6B. Poly-Lys-Ser, Poly-Lys, Oligo-Arg-Ser, Oligo-108-124, Oligo-69-84, Oligo-(115+116-del), fibronectin, ovalbumin, and LDL (1 g/liter each) as well as ^{125}I-rPDGF(c-sis) and human serum were chromatographed on a column (16 × 40 mm) utilizing the HPLC gradient former and pump. The sample volume was 1 ml, flow rate 0.5 ml/min, and the fractions were 1 ml. Unbound material was eluted in 50 ml PBS and bound material in a gradient (total 50 ml) of sodium chloride (0.15 to 2 *M*) in phosphate buffer (pH 7.4).

TABLE 1

BINDING OF TEST SUBSTANCES TO HEPARIN-SEPHAROSE CL 6B

	Dissociating Concentration of NaCl, *M*
Ovalbumin	Unbound[a]
Oligo-69-84	0.15
LDL	0.3
Fibronectin	0.4
^{125}I-PDGF(c-sis)	0.5
Oligo-(115 + 116-del)	0.7
Oligo-Arg-Ser	0.7
Poly-Lys	0.8
Oligo-108-124	0.9
Poly-Lys-Ser	1.8
RF I	0.4
RF II	1.3

[a] Did not bind to the column in 0.15 *M* NaCl.

Eluates were monitored for protein by absorbancy in a spectrophotometer at 280 or 226 nm as appropriate and radioactivity by gamma counting. The salt concentration of eluted fractions was determined by refractometry. The refractometer was standardized against known concentrations of phosphate buffered NaCl.

Cell culture conditions. The isolation, characterization, and culture conditions of untransformed mycoplasma-free hASMC have been described previously (Fager et al., 1988, 1989, 1991). Briefly, proliferation of secondary cultures was induced in supplemented Waymouth's MB 752/1 medium with 10% (vol/vol) human serum and 10% fetal bovine serum for 3 days and then growth-arrest by incubation in serum-free medium containing 1% (wt/vol) BSA for another 3 days before test experiments.

The competition between defined test components and growth factors for heparin was investigated in competition experiments, with hASMC proliferation indices as biological endpoints. The medium was changed to test medium with 10% human serum and 0.05 g/liter heparin or to control medium with 1% BSA and supplemented in duplicate or triplicate cultures with different amounts of test substance. This concentration of heparin has been found to induce half-maximal inhibition of proliferation among our hASMCs in medium with 10% human serum (Fager et al., 1988). The possibility that the oligopeptides in the presence of the unretained fraction from Heparin-Sepharose chromatography (UF) stimulated thymidine incorporation directly was also investigated.

In all experiments, cells were seeded sparsely (500 cells/cm^2) to minimize the effect of cell-to-cell interactions. For DNA measurements, the cells were seeded in 35-mm petri dishes, and for determinations of thymidine indices in Lab-Tec slides. Media were changed every third day.

Determination of thymidine index and total DNA. The thymidine index was determined after 3 days in test medium by incubating the cultures in the presence of [6-^{3}H]thymidine (2 mCi/liter final concentration) for another 24 h before preparation for autoradiography as previously described (Fager et al., 1988).

Total DNA was determined by an in situ DNA assay (Johnson-Wint and Hollis, 1982) in cultures grown in petri dishes in test media for 6 days before DNA measurements.

Results and Discussion

Several test peptides bound to the Heparin-Sepharose column at physiologic concentrations of sodium chloride (Table 1). As will be

seen, our results suggested that only two of them competed effectively with serum mitogen(s) for heparin in culture medium to increase thymidine incorporation into hASMCs.

The lack of endogenous production of PDGF as well as the critical dependence on exogenous PDGF for the transition from a state of quiescence and differentiation into one of proliferation and dedifferentiation made our human ASMCs (Fager et al., 1988, 1989, 1991) suitable as target cells for studies on PDGF effects in vitro. The inhibition of proliferation and the redifferentiation that we observed in the presence of heparin were found to be due to a reversible binding and inactivation of PDGF by heparin in the companion article (Fager et al., 1991).

In preliminary experiments, we tested the possibility that highly positively charged peptides might compete with PDGF for binding to heparin. In dilutions between 10^{-4} and 10^{-11} *M*, Poly-Lys-Ser but neither Oligo-Arg-Ser nor Poly-Lys increased DNA synthesis in hASMCs grown in the presence of serum and a 50% inhibitory concentration of heparin (data not shown). The maximal effect of Poly-Lys-Ser occurred reproducibly at about 10^{-6} *M* concentration of the peptide. All three peptides did, however, bind to Heparin-Sepharose at higher salt concentrations than did rPDGF(csis) (Table 1). Among the other control peptides, ovalbumin did not bind to Heparin-Sepharose in 0.15 *M* NaCl and LDL and fibronectin dissociated at salt concentrations that were lower than that of rPDGF(csis) (Table 1). In no experiment did any one of them increase thymidine incorporation in the test system at concentrations between 10^{-4} and 10^{-11} *M* (data not shown).

This suggested: a) that ionic interactions between PDGF and heparin did not alone explain the inhibition of cell proliferation, b) among highly charged basic and hydropathic amino acids residues, lysine was more important than arginine; and c) uninterrupted sequences of lysine were ineffective in the competition with serum mitogen(s) for heparin. Poly-Lys-Ser had a ratio of lysine:serine of 3:1. This suggested that sequences of [-Lys-Lys-X-Lys-] in PDGF might indeed be involved in a specific binding to heparin-like GAGs, as suggested for other peptides by Cardin and Weintraub (1989). They ascribed the affinity for heparin to sequences of [-X-B-B-X-B-X-] where [B] is a basic and [X] a hydropathic amino acid residue. Our preliminary results suggested that among such [B] residues, lysine was more effective than arginine in the binding of PDGF to heparin. They also suggested additional importance of the [X] residues.

The transcription of the PDGF A-chain is subject to alternative splicing at the sixth exon giving two transcripts encoding A-chains of different carboxylic termination with a 15 amino acid extension in the longer isoform (Betsholtz et al., 1986; Rorsman et al., 1988; Bonthron et al., 1988). The regulation and significance of this alternative splicing is unknown. It is longer isoform, the human PDGF A-chain contains two highly charged basic amino acid sequences [-Lys^{74}-Lys^{75}-Pro^{76}-Lys^{77}-Leu^{78}-] and [-Lys^{115}-Lys^{116}-Arg^{117}-Lys^{118}-Arg^{119}-]. These domains constitute tentative binding sites for heparin-like GAGs. The shorter transcript encodes an A-chain isoform that contains only the former domain (residues 74–78). A similar sequence is found in acidic FGF (residues 9–12) where it constitutes one of two sequences implicated in heparin binding previously (Esch et al., 1985 a, b). The PDGF B-chain contains no such sequence in its *primary* structure.

To test these tentative binding sites, we prepared synthetic oligopeptides corresponding to amino acid residues 69–84 and 108–124 of the A-chain as well as two control peptides. The purity of the peptides was verified with analytical HPLC, and the correct amino acid sequence confirmed by amino acid sequencing. CD spectra of the peptides showed no sign of alpha-helical or beta-sheet structures in solution with or without heparin.

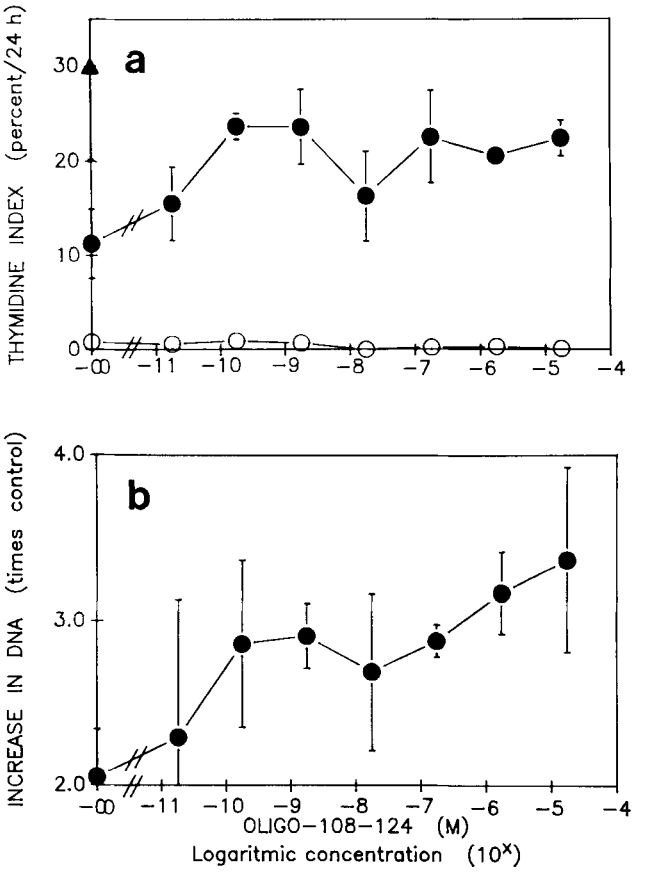

FIG. 1. Effect of Oligo-108-124 on hASMC grown in medium with human serum (10%) and heparin (0.05 g/liter) (*closed circles*) and in serum-free medium without heparin (*open circles*) on thymidine index (*a*) and DNA content (*b*). *Triangle* indicates the values of cultures grown in 10% serum without heparin. Values from one set of experiments are given as mean ± SD of duplicate determinations.

When Oligo-108-124 was added in increasing concentrations to hASMC grown in medium with a constant content of heparin and serum, the thymidine index increased reproducibly to a plateau level already at 10^{-10} *M* of the peptide (Fig. 1 *a*). These results were confirmed in similar experiments by determination of total DNA (Fig. 1 *b*). This concentration of Oligo-108-124 was almost equimolar to that at which PDGF caused maximum mitogenic response in hASMCs (Fager et al., 1991). Considering that the system was balanced at a heparin concentration causing about 50% inhibition of serum mitogen(s) (cf. Fig. 1 and 3), we concluded that Oligo-108-124 and PDGF either bound to heparin with commensurate affinity or that Oligo-108-124 itself was able to bind to and stimulate the PDGF receptor directly and as effectively as PDGF. The lack of stimulation by Oligo-108-124 in serum-free medium (Fig. 1 *a*) did not exclude the latter possibility because we have previously found that the proliferative response of hASMC to PDGF depends on additional serum factors; together but not alone, UF

and PDGF stimulated hASMC proliferation (Fager et al., 1991). The thymidine index of cultures grown in UF increased significantly when rPDGF-AA or rPDGF-BB were added but not when Oligo-108-124 or Oligo-(115+116-del) were added (Fig. 2). As found previously (Fager et al., 1991), the mitogenic effect of UF was clearly less than that of the source-serum before chromatography and only slightly higher than that of serum-free medium. The present results indicated that the monomeric oligopeptides did not themselves stimulate PDGF receptors.

In repeated experiments, Oligo 69-84 had no effect on heparin-induced inhibition of thymidine incorporation in the cell assay (Fig. 3 *a*). In Oligo-69-84, the three lysine residues are interrupted by a proline, whereas they are interrupted by arginine in Oligo-108-124. In contrast to arginine, proline would introduce a beta-turn in this sequence and decrease its charge and hydrofilicity, changes that were likely to alter the affinity of the peptide for heparin. Indeed, Oligo-69-84 dissociated from Heparin-Sepharose at column chromatography already in 0.15 *M* NaCl, whereas Oligo-108-124 dissociated at 0.9 *M* and rPDGF(c-sis) at 0.5 *M* NaCl (Table 1).

The importance of the sequence [-Lys^{115}-Lys^{116}-Arg^{117}-Lys^{118}-Arg^{119}-] in Oligo-108-124 was further investigated by deleting lysine residues 115 and 116 in the synthesis of another oligopeptide, Oligo-(115+116-del). The deletion of these lysine residues reduced the ionic interaction of the sequence 108-124 with heparin only from 0.9 to 0.7 *M* NaCl. Nevertheless, Oligo-(115+116-del) failed completely and repeatedly to restore heparin-induced inhibition of thymidine incorporation in hASMCs (Fig. 3 *b*).

Thus, our results strongly suggest that the interaction between PDGF and heparin-like GAGs is specific and that the amino acid sequence [-Lys^{115}-Lys^{116}-Arg^{117}-Lys^{118}-Arg^{119}-] plays a central role in this specificity. For heparin binding, adjacent amino acids within this region may, however, also be important. We cannot exclude the possibility that other domains of the PDGF monomers may bind to heparin. The fact that ^{125}I-rPDGF(c-sis) bound to Heparin-Sepharose and that Oligo-108-124 was unable to relieve the inhibitory effect of heparin completely supported the possibility of additional binding sites. Our results do, however, suggest that the alternative splicing of PDGF A-chain mRNA may be biologically important, because only the long PDGF A-chain isoform (Betsholtz et al., 1986; Rorsman et al., 1988; Bonthron et al., 1988; Matoskova et al., 1989) contains the critical sequence described here. This domain of the A-chain has also been implicated in its more efficient secretion (LaRochelle et al., 1990) and shown to be a nuclear targeting signal (Maher et al., 1989).

Definite conclusions about the importance of individual amino acid residues within the C-terminal sequence of the long A-chain isoform or of other domains of the two PDGF monomers for heparin binding must await the use of recombinant mutants. The oligopeptides used here never resumed a structure of higher order, making impossible a detailed understanding of the behavior of full-length peptide variants.

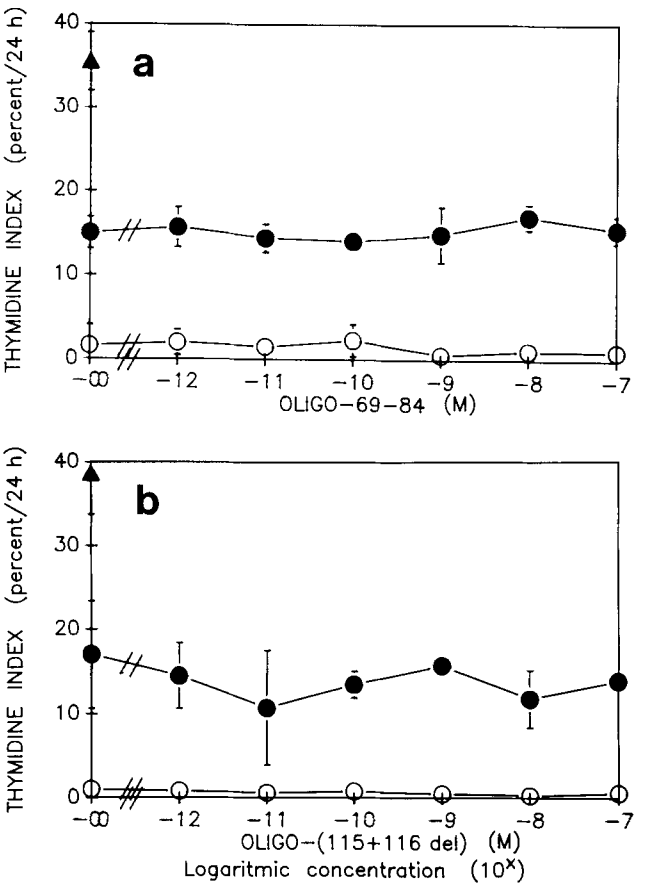

FIG. 3. Effects of Oligo-69-84 (*a*) and Oligo-(115+116-del) (*b*) on thymidine index of hASMC grown in medium with human serum (10%) and heparin (0.05 g/liter) (*closed circles*) and in serum-free medium without heparin (*open circles*). *Triangles* indicate the values of cultures grown in 10% serum without heparin. Values from one set of experiments are given as mean ± SD of triplicate determinations.

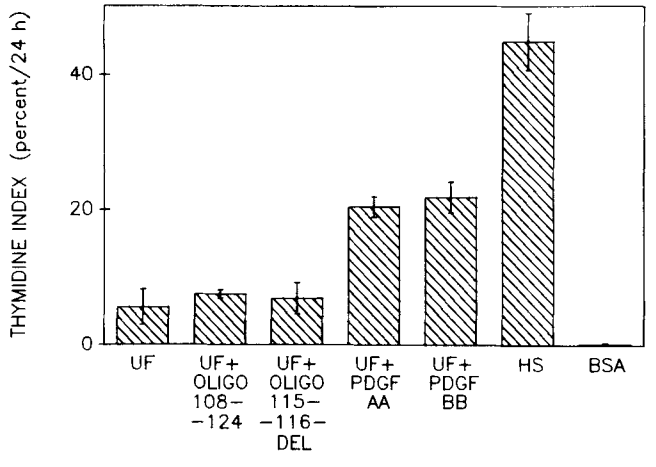

FIG. 2. Effects of Oligo-108-124 (10^{-7} *M*) and Oligo-(115-116-del) (10^{-7}*M*) on thymidine index of hASMC grown in medium with the unretained serum fraction (UF) from Heparin-Sepharose CL 6B chromatography (7.4 g/liter). Cultures grown in serum-free medium (BSA, 10 g/liter) and in human serum (HS, 7.1 g/liter) served as controls. The results with the oligopeptides are compared with those of recombinant PDGF AA and BB homodimers (3×10^{-10} *M*). Values from one experiment are given as mean ± SD of duplicate determinations.

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