

Poly-L-lysine/Heparin Stimulates Angiogenesis in Chick Embryo Chorioallantoic Membrane

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The effects of heparin on angiogenesis are controversial, with some studies claiming stimulatory and other studies claiming inhibitory effects. Since heparin in human plasma is complexed with basic peptides and proteins, we studied the angiogenic effect of complexes resulting by mixing poly-L-lysine (a basic heparin-binding polypeptide) and heparin. Angiogenesis was investigated by chorioallantoic membrane assay. In specimens treated with PBS (negative control), or poly-L-lysine, no significant vascular reaction was detectable. Heparin induced only moderate angiogenic response. However, neutral complexes purified from a mixture of poly-L-lysine and heparin (20/1, w/w) induced a very strong angiogenic response. These results demonstrate that the angiogenic effect of heparin was associated with neutralization of electric charge when the polysaccharide was complexed with a basic peptide. © 2002 Elsevier Science

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Heparin has diverse effects on angiogenesis with some studies claiming an angiogenic effect while others demonstrating an antiangiogenic effect (1). Heparin was shown to bind both angiogenic growth factors (2) and anti-angiogenic proteins (3), favoring and stabilizing the association with their respective receptors.

However, since circulating heparin is tightly associated with a variety of proteins and peptides that mask its anticoagulant activities (4, 5), one could argue that neutralization of negative charges per se might provide heparin with other biological roles independently of the activity of the binding peptide–proteins. Thus, we studied the angiogenic effect of neutral complexes purified

Abbreviations used: CAM assay, chorioallantoic membrane assay; PBS, phosphate-buffered saline.

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from mixtures of poly-L-lysine (a basic heparin-binding polypeptide with no angiogenic activity) and heparin.

MATERIALS AND METHODS

Materials. [³H]heparin (sp act 440 μCi/mg, molecular mass 6.0–20.0 kDa) was from New England Nuclear (Boston, MA). Heparin EP 756 (average molecular mass 12.9 kDa) was from Opocrin Research Laboratories (Modena, Italy) (6). Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), collagenase (EC 3.4.24.3), and poly-L-lysine (molecular mass 78.0 kDa) were from Sigma Chemical Co. (St. Louis, MO). Papain (EC 3.4.22.2) was from Calbiochem (La Jolla, CA); pepsin (EC 3.4.23.1) was from Boehringer Mannheim (Mannheim, Germany); heparinase I (from *F. heparinum*, EC 4.2.2.7) was from Seikagaku Kogyo (Tokyo, Japan). DEAE-Sephacel was from Pharmacia (Uppsala, Sweden); Carboxymethyl cellulose CM-52 was from Whatman (Maldstone, England). Dialysis membranes (molecular mass cutoff 3.5 kDa) were from Spectrum (Breda, The Netherlands). Phosphate-buffered saline (PBS), Prostaglandin E1 (PGE1) and other common reagents were from Sigma Chemical Co. (St. Louis, MO). Gelatin sponges (Gelfoam) were from Upjohn Co., (Kalamazoo, MI).

Methods. To rule out possible contamination of commercial heparin by angiogenic factors, heparin was submitted to exhaustive proteolysis and purified by ion-exchange chromatography, before being mixed with poly-L-lysine. Papain (1 mg/ml) was added to a heparin solution (10 mg/ml) in 0.05 M Tris–HCl, pH 7.4, for 24 h at 60°C. Subsequently, the sample was boiled and, at time intervals of 24 h, the following enzymes were added sequentially: trypsin, chymotrypsin, collagenase, and pepsin, each at final concentration of 1 mg/ml. Each digestion was performed for 24 h at 37°C and the sample was boiled before addition of each protease. After proteolysis, the sample was centrifuged at 15,000g for 10 min, and heparin was purified by passage on a DEAE-Sephacel column (1 × 3 cm), equilibrated in 0.05 M sodium acetate buffer, pH 4.0. The column was washed with 50 ml of 0.3 M NaCl in the same buffer, and then eluted with 2.0 M NaCl. The 2.0 M NaCl fraction was dialyzed and lyophilized; this fraction contained more than 95% of initial heparin. In the present study this fraction (termed protease-treated heparin) was subsequently mixed with poly-L-lysine at ratios of 1/1 and 1/20 (w/w; final protein concentration 1 mg/ml). These samples were applied to a DEAE-Sephacel column (1 × 3 cm), equilibrated in 0.05 M Tris–HCl, pH 7.4. The column was washed with 100 ml of the same buffer, and the fraction unretained by DEAE-Sephacel was loaded onto a CM-52 column (1 × 3 cm). The columns were eluted with increasing concentrations of NaCl in 0.05 M Tris–HCl, pH 7.4. The neutral, unretained material by both DEAE-Sephacel and CM-52 resins, was dialyzed and assayed for its angiogenic properties. In

TABLE 1

Ion-Exchange Chromatography of [³H]Heparin in the Absence or in the Presence of Poly-L-Lysine

	³ H radioactivity (%) retained by	
	DEAE-Sephacel	CM-52
Heparin	98.2 ± 0.2	—
Poly-L-lysine/heparin 1/1	97.1 ± 0.4	—
Poly-L-lysine/heparin 20/1	55.3 ± 0.5	25.2 ± 2

Note. Poly-L-lysine was mixed with standard heparin at a ratio of 1/1 and 20/1 (w/w) at a final protein concentration of 1 mg/ml; 1×10^6 dpm of [³H]heparin was added as a marker. These samples were submitted to ion-exchange chromatography on a DEAE-Sephacel column and the unretained material was loaded onto a CM-52 column. ³H radioactivity retained by DEAE-Sephacel and CM-52 was expressed as % of the total ³H radioactivity of the starting sample. The values are means ± SEM for 2 experiments.

other experiments, mixtures of poly-L-lysine and standard heparin at ratios 1/1 or 20/1, containing 1×10^6 dpm of [³H]heparin as tracer, were submitted to sequential ion-exchange chromatography as above, and radioactivity eluted at different ionic-strength was measured by liquid scintillation counting (Table 1). In other experiments, commercial heparin was treated exhaustively with heparinase I as previously described (7).

For chorioallantoic membrane (CAM) assay, white Leghorn chicken eggs were incubated under routine conditions (constant humidity and 37°C) and a square window was opened in the egg shell at day 3 of incubation, after removal of 3.5 ml of albumen to detach the shell from the developing CAM. The window was sealed with a glass of the same size, and the eggs were returned to the incubator. Gelatin sponges were cut to a size of 1 mm³ and placed on the top of the CAM at day 8 under sterile conditions (8). The sponges were then absorbed with 5 μl of compounds to be tested. Sponges containing PBS were used as negative controls. CAMs were examined daily and photographed *in ovo* at day 12. The angiogenic response of the CAMs was scored qualitatively as follows: ++++ (very strong), +++ (strong), ++ (significant), + (moderate), +/- (slight), or - (no effect) (9).

RESULTS

To purify neutral poly-L-lysine/heparin complexes, first we studied the separation pattern of mixtures of poly-L-lysine and heparin (ratio 1/1 and 20/1) on DEAE-Sephacel and CM-52 resins using [³H]heparin as tracer (Table 1). Mixing poly-L-lysine and heparin did not cause formation of microprecipitates as demonstrated by recovery of 95% of [³H]heparin in the supernatant after centrifugation. The ratio poly-L-lysine/heparin significantly influenced heparin association with DEAE-Sephacel. The ratio poly-L-lysine/heparin 1/1 allowed recovery of [³H]heparin at higher salt concentration (0.6 and 1.0 M NaCl) than the mixture poly-L-lysine/heparin 20/1 (0.4, 0.6 M). Thus, a ratio poly-L-lysine/heparin 1/1 was not sufficient to entirely neutralize the negative charges on heparin molecule as demonstrated by strong binding to DEAE-Sephacel. However, approximately 45% of [³H]heparin was unre-

tained by DEAE-Sephacel when the ratio poly-L-lysine/heparin was 20/1. This unretained fraction was subjected to CM-52 resin separation, and about 55% of this material bound the acidic resin. From CM-52, ³H radioactivity eluted with a peak at 1.0 M NaCl concentration. Thus, mixing poly-L-lysine with heparin at a ratio 20/1 caused the formation of neutral complexes that could be purified by passage on the two columns; about 20% of heparin was recovered in neutral complexes. For CAM assay, we prepared poly-L-lysine/heparin mixtures using protease-treated heparin.

Standard heparin (untreated) caused moderate angiogenic response as previously reported (8) (Fig. 1d; Table 2); heparin that had been submitted to exhaustive proteolysis and purified, evoked a similar response (Fig. 1c; Table 2, protease-treated heparin). However, after digestion with heparinase I, heparin lost its proangiogenic activity (Table 2, heparinase-treated heparin). Poly-L-lysine did not significantly increase the basal vascularization observed in negative controls (Fig. 1b; Table 2). The neutral complexes, i.e., those unretained from both resins, elicited a very strong angiogenic response (Table 2, poly-L-lysine/heparin, neutral complexes). Blood vessels with an irregular course and frequent branching were present at day 12; the gelatin sponges were surrounded by allantoic vessels that developed radially toward the implant in a "spoked-wheel" pattern. Tortuous vessels infiltrated the sponge (Fig. 1f). Angiogenic response was even stronger than the response induced by PGE1 (1mg/ml, i.e., 5 μg/sponge), a powerful stimulator of angiogenesis (10) (Fig. 1e; Table 2). It is worth noting that a mixture of poly-L-lysine/heparin at a ratio 20/1 (i.e., the mixture from which the neutral complexes were subsequently separated by sequential ion-exchange chromatography) induced a significant angiogenic response (Table 2, mixture poly-L-lysine/heparin 20/1); these results could be interpreted as if the native mixture contained a smaller percentage of neutral complexes in comparison with the material unretained from both resins that was enriched of neutral complexes. Consistently, a native mixture of poly-L-lysine/heparin at a ratio 1/1 (i.e., a mixture that did not give rise to formation of neutral complexes (Table 1) did not elicit any significant vascular response (Table 2, mixture poly-L-lysine/heparin 1/1).

DISCUSSION

Our results suggest that the angiogenic effect of heparin is enhanced, or evidenced, when the negative charges on its surface are masked. Thus, the nature of the heparin-binding peptides does not appear to be essential in this respect as long as they neutralize heparin negative charges. It is also conceivable that neutral protein/heparin complexes are randomly formed when commercial heparin preparations are as-

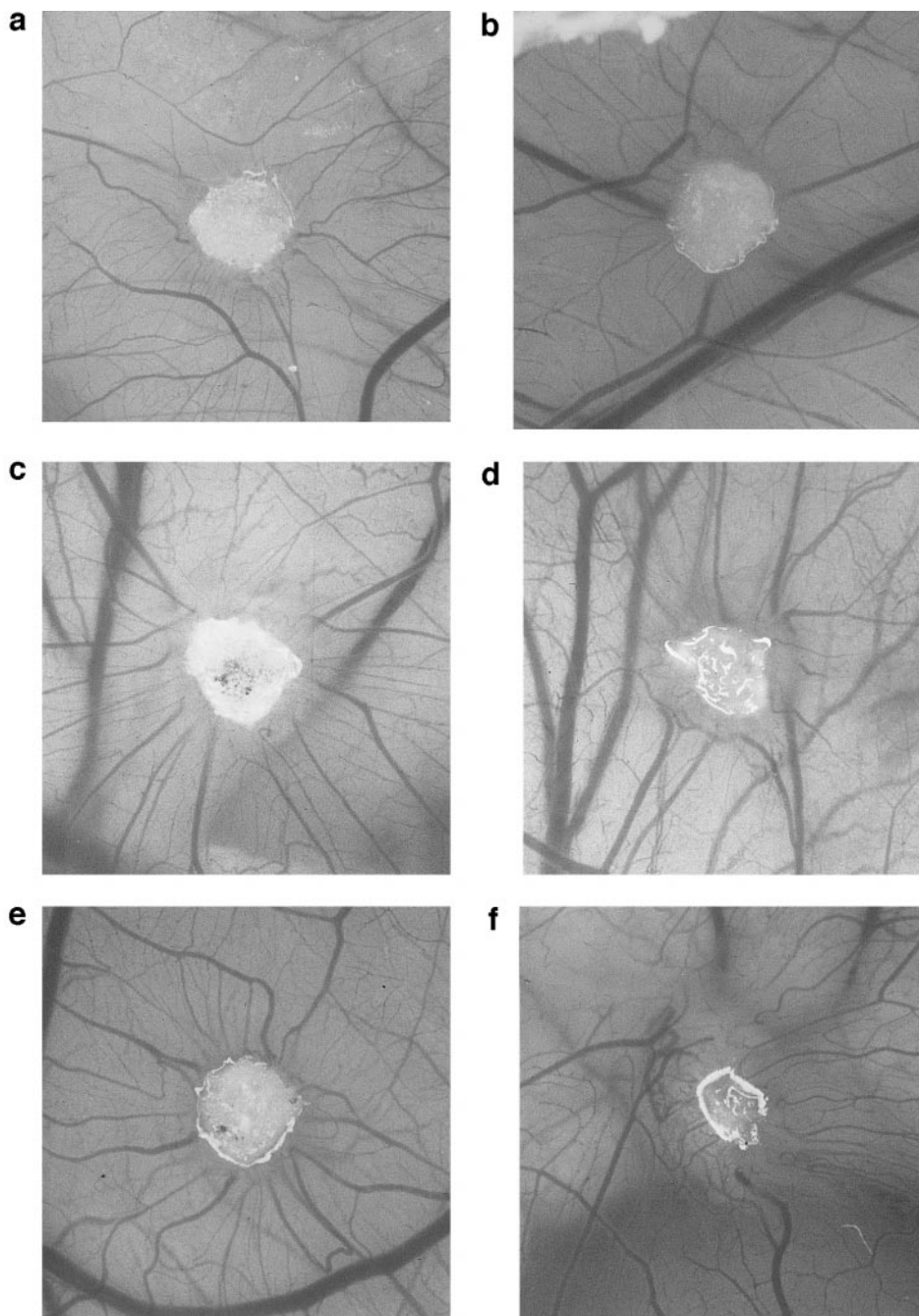


FIG. 1. Angiogenic response of the CAM after implantation of gelatin sponges adsorbed with different substances. (a) Negative control, basal vascularization. (b) Poly-L-lysine (1 mg/ml). The compound did not significantly increase basal vascularization observed in negative controls. (c) Protease-treated heparin (1 mg/ml). The compound induced a moderate angiogenic response. (d) Standard heparin (untreated) (1 mg/ml). The compound induced a moderate angiogenic response. (e) PGE1 (1 mg/ml). PGE1, used as positive control, induced a strong angiogenic response with numerous allantoic vessels developing radially toward the implant. (f) Neutral complexes poly-L-lysine/heparin. The complexes induced a very strong angiogenic response with vessels more numerous than those observed in positive controls.

sayed in different experimental conditions, especially *in vivo*; differences in the formation of these complexes could be held accountable for the observed variety of often contradictory effects of heparin on angiogenesis, and for the unreproducibility of some results (10, 11).

One could speculate that the supramolecular assembly of endogenous heparin, either with phospholipids (12) or proteins (5) is a means to modulate its biological functions. Heparin administered as an anticoagulant drug might be partly complexed with plasma proteins,

TABLE 2

Qualitative Evaluation and Score for Angiogenic Response in Chorioallantoic Membrane Assay

Specimen	Qualitative angiogenic response	Score
Negative control (PBS)	No effect	–
Poly-L-lysine	Slight	+/-
Standard heparin (untreated)	Moderate	+
Protease-treated heparin	Moderate	+
Heparinase-treated heparin	No effect	–
Mixture poly-L-lysine/heparin 1/1	Slight	+/-
Mixture poly-L-lysine/heparin 20/1	Significant	++
Poly-L-lysine/heparin (neutral complexes)	Very strong	++++
Positive control (PGE1)	Strong	+++

resulting in the formation of angiogenic complexes; this “side effect” might be responsible for the observed revascularization occurring after prolonged heparin treatment (13).

As far as the mechanism of action of peptide/heparin complexes in stimulating angiogenesis is concerned, one could speculate that these complexes might work at the level of the plasma membrane (14) or be internalized (15) and affect gene expression in endothelial cells. Thus, it was recently demonstrated that heparin could regulate gene expression (16) and stabilize, potentiate and transport a growth factor into the nucleus (17).

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