Short Communication

Adhesion inhibition "*in vitro*" by heparin derivatives correlates with their activity on angiogenesis in mice

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Abstract

Heparin (HEP) play an important role in angiogenesis. Inhibition of matrix degrading enzymes and binding to adhesion molecules are some of the interaction mechanism. The bleeding risk and the need of parenteral administration, restrict the therapeutic use of HEP as angiogenesis modulator; presently derivatives, with different pharmacokinetic and anticoagulant properties, are available. In this study the "*in vitro*" anti-adhesion activity, tested with PMNs and endothelial cells, was compared with the effect on angiogenesis, evaluated in Matrigel implanted mice. Some HEP derivatives, with low anticoagulant activity, showed a significant angiogenesis inhibition. A positive correlation between adhesion inhibition *in vitro* and anti-angiogenesis effect *in vivo* was found suggesting that the interaction with adhesion molecules by HEP derivatives play a relevant role in the angiogenesis control.

Keywords: angiogenesis • adhesion • heparin • sulphated glycosaminoglycans

Introduction

Many diseases are dominated by an anomalous or excessive angiogenesis. Tumor growth and metastasis formation are also angiogenesis dependent. Control of angiogenesis would be of interest if safe and effective angiogenesis inhibitors would be available.

Heparin play an important role in angiogenesis and early data showed very promising antiangio-

genic effects especially in combination with steroids; unfortunately further studies did not confirm these good results.

Moreover the bleeding risks and the need of parenteral administration, reduced the interest on heparin as angiogenesis modulator.

In the last years various derivatives of heparin, with different pharmacokinetic and anticoagulant properties have been produced and some of them seems to have interesting antiangiogenic activities in experimental models [1, 2].

In order to characterize the action of heparin derivatives in the adhesion processes, our group

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studied a series of de-sulphated heparin derivatives [3]. These products present relevant characteristic having almost no anticoagulant activity and being absorbed, at a significant extent, orally. Inhibitory results on adhesion were obtained and, considering the important role played by adhesion molecules in angiogenesis, prompted us to evaluate the anti-angiogenic properties in correlation with the anti-adhesion activity of desulphated derivatives of heparin vs. heparin itself.

The results of these experiments are further presented.

Materials and methods

“*In vitro*” testing

Heparin and heparin derivatives

Heparin, 2-O-desulphated-heparin (2-O-des-Hep), N-desulphated-heparin (N-des-Hep), and 2-O-N-desulphated-heparin (N-O-des-Hep) were kindly supplied by Alfa-Wassermann, Bologna, Italy.

Cell preparations

Human umbilical vein endothelial cells (HUVECs), passage 3-4, were cultured in multiwell plates and used for adhesion and Platelet Activating Factor (PAF) synthesis experiments when forming a tightly confluent monolayers.

Human PMNs were prepared from healthy donors by differential centrifugation and gelatine sedimentation, followed by osmotic shock. Smears of stained cells (Giemsa) showed 85-90% PMNs; the viability of PMNs (trypan blue exclusion) was always greater than 95%. Approximately 55 μCi of In-oxine (Amersham, UK) was added dropwise to 2 ml of PMN suspension and allowed to incubate at room temperature for 10 min. After centrifugation at 1400 g for 10 min, PMNs were resuspended in Tris-buffered Tyrode's solution containing 0.5 g human serum albumin (HSA) (Sigma). The labelling efficacy counted in a gamma counter was about 90%.

PMNs adhesion to HUVECs

HUVECs were washed with M199 medium (Sigma) containing 0.5% HAS and incubated at 37°C for 15 minutes with 10^{-8} M PAF or with control buffer. After incu-

bation, the medium was removed, the monolayers were washed twice and In-labeled PMNs were added. Following the incubation periods, PMNs were removed, HUVECs were washed twice with the incubation medium and the adherent radiolabeled PMNs were solubilized for 10 minutes with 1 M NaOH containing 1% SDS. Finally the net percent adhesions were determined by counting the radioactivity of the adherent PMN fraction and calculating the percent of total radioactivity added. Two sets of experiments were carried out: in each set of experiments either HUVECs or PMNs were pre-incubated, before their co-incubation, with 0, 0.1, 1, 10, or 100 $\mu\text{g/ml}$ of heparin or its desulphated derivatives for 10 minutes at room temperature.

“*In vivo*” testing

Animals

Nine-week-old male Swiss mice were obtained from the animal facilities of the National Institute for Pharmaceutical Chemistry and used for the experiments. The ethical committee of the institute approved the study protocol.

Matrigel inoculation

According to the method described by Passaniti *et al.* 1992 [4], 0.5 ml of matrigel (Becton Dickinson Labware, USA), at 10 mg protein ml^{-1} , containing VEGF, 30 ng ml^{-1} ; (Sigma) and heparin, 58 units ml^{-1} . (Sigma) was injected s.c. on the dorsal surface.

Heparin and heparin derivatives administration

Heparin, 2-O-des-Hep, N-des-Hep, N-O-des-Hep, dissolved in normal saline solution (0.9%) at 10 or 1 mg/ml, were administered *i.v.*, 1.0 mg (solution 10 mg/ml) or 0.1 mg per animal (solution 1 mg/ml), twice a day for 3 days. For each treatment and concentration or control 6 mice were tested.

Angiogenesis evaluation

Three days later, the mice were sacrificed under ether anesthesia, and matrigel was collected from surrounding connective tissue. After digestion with 0.02% collagenase (Sigma), the haemoglobin content of the matrigel was determined by Hemoglobin Test (Sigma). The results, as hemoglobin content, were calculated normalized to the amount of matrigel removed.

Results

“*In vitro*” PMNs - HUVECs adhesion tests

The experimental results resumed in Fig. 1 clearly show that heparin and its desulphated derivatives have inhibitory effects on PMNs adhesion, when the PMN's are pre-treated with these compounds. The effects are of different degree of intensity for each of the tested compounds and for each compound are dose dependent.

Heparin gave the best results with significant inhibitions, $p < 0.05$, at all tested concentrations.

Desulphated derivatives shown at different extent, less anti-adhesion effects: the O-des-hep had a statistically significant effect ($p < 0.05$) for all concentrations ≥ 1 mg/l; similar results were obtained with the N-des-Hep that presented statistically significant effects vs. control ($p < 0.05$) for all concentrations ≥ 1

mg/l; however in comparison with O-des-Hep, the N-des-Hep shown a non-significant lower activity. Although that the de-sulphation degree is almost identical between the two derivatives is evident that the position of the sulphate group in the heparin molecules, or the presence of free amino groups left by the N-desulphation play a certain role in the modulation of the anti-adhesion properties. The N-O-des-Hep, presented a significant inhibition effect vs. control only for 10 folds higher dosages that that required for the only O- or only N- de-sulphated derivatives (statistically significant effect vs. control, $p < 0.05$, only for dosages over 10 mg/l), being so far the less potent of all the tested compounds.

The pretreatment of HUVECs with heparin and its derivatives, Fig. 2, did not show any inhibitory effect on adhesion by these compounds, at any of the tested concentration; the comparison of these results with the previous-ones clearly shows that heparin and its derivatives interact with adhesion

Fig. 1 Adhesion inhibition by Hep and derivatives - PMNs pretreatment.

Fig. 2 Adhesion inhibition by Hep and derivatives - HUVECs pretreatment.

Table 1 *In vivo* inhibition of angiogenesis.

	Heparin	N-des-Heparin	O-des-Heparin	N-O-des-Heparin
	0.1 mg	0.1 mg	0.1 mg	0.1 mg
MEAN	1.50	2.20	3.20	4.30
ST.DEV.	0.77	0.70	1.34	1.91
p (vs control)	0.0001	0.0008	0.0458	0.3820
	1.0 mg	1.0 mg	1.0 mg	1.0 mg
MEAN	1.20	1.60	2.80	3.00
ST.DEV.	0.74	0.66	1.22	1.11
p (vs control)	0.0001	0.0002	0.0114	0.0286

The angiogenesis was evaluated as haemoglobin content in matrigel implants. The mean and standard deviation of 6 experiments per each point are reported. Student "t" test was used for statistical comparisons. The control mean was 4.50 and Standard Deviation was 1.05.

molecules, or receptor of adhesion molecules, that are presents on the PMNs surface but not on the HUVECs.

“*In vivo*” matrigel angiogenesis test

The results obtained from the “*in vivo*” experiment, presented in Table 1 and resumed in Fig. 3 confirmed that, in our experimental conditions, heparin exert a significant anti-angiogenic effect giving an hemoglobin content in matrigel < 30 % of untreated control ($p < 0.05$ for both dose tested).

However it is noteworthy that half of the mice treated with the highest heparin concentration presented bleeding complications ; such effects were not observed in any other treated group as well as in controls.

The results obtained with O-des-Hep and N-des-Hep were statistically significant ($p < 0.05$, vs. control) for both dosages administered, while for N-O-des-Hep results where less relevant ($p < 0.05$, only for the highest dose tested “*in vivo*”, while for the lowest dose, the effect had no statistical significance $p = 0.38$).

Fig. 3 Angiogenesis inhibition by Hep and derivatives - *In vivo* experiments.

The anti-angiogenic activity paralleled quite well the anti-adhesion results obtained on PMNs-HUVECs “*in vitro*”, suggesting that exist a good correlation between the chemical structure of these compounds and these two biological activities (anti-adhesion and anti-angiogenesis effects).

Discussion

The results obtained “*in vitro*” confirm the important role of heparin in modulating adhesion processes. At a less extent also partially desulphated heparin derivatives still present anti-adhesion effect. The effects were similar for the O- and N- des-Hep and less relevant for the N-O-des-Hep. Analogous results were obtained on low-sulphated heparan sulphate derivatives by another research group, Hahnenberger R et Al. 1993 [5]. Interestingly this research group found an higher activity in oligosaccharide containing iduronic acid residues ; this exuronic acid is typically present in heparin more then in heparan sulfate. The results obtained from these “*in vitro*” experiments, put also in evidence the importance of the adhesion molecules or adhesion molecules receptors present on different kind of cells. The absence of certain adhesion molecules from HUVEC, that were present on the PMN cells, has completely modified their adhesion behaviour. While for all tested compounds (heparine and the three tested derivatives), at different concentrations (compound dependent), a statistically significant inhibition of the adhesion was obtained when PMN cells were pre-incubated, in the experiments in which HUVEC were pre-incubated, no significant effect was encountered.

The “*in vivo*” results put in evidence a striking correlation between the antiadhesion efficacy of heparin or its different desulfation derivatives and the antiangiogenic activity as measured by the hemoglobin content in matrigel.

Such strict result correlation suggests that adhesion inhibition play a primary role in the antiangiogenic activity of heparin and its derivatives.

The role of adhesion in angiogenesis is clearly defined and doesn't require other comments.

In the past various researcher observed a pro-angiogenic effect of heparin; a large part of this effect can be connected to the binding properties of heparin to various growth factors (e.g. bFGF, PDGF); more recently have been described the binding of heparin to an EGF-like growth factor by Raab et al. 1997 (6), determining a local accumulation of it in an active form and promoting angiogenesis.

In conclusion, our results obtained with these desulphated heparin derivatives suggest a potential therapeutic role as anti-angiogenic drug for this category of molecules. These products and other similar molecules present over heparin the advantage of an easier way of administration (i.e. orally) for prolonged periods and without the bleeding risk typically observed with heparin by parenteral administration.

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