J. Pharm. Pharmacol. 1993, 45: 1009–1011
Communicated March 3, 1993

© 1993 J. Pharm. Pharmacol.

Influence of heparin on the chemotactic activity of human thrombin

A. MORIN*†, M. MARCHAND ARVIER*†, J. C. HUMBERT*, F. DOUTREMEPUICH†, C. VIGNERON*†, *Centre Régional de Transfusion Sanguine et d'Hématologie, 54511 Vandoeuvre Les Nancy Cedex, and †Faculté des Sciences Pharmaceutiques et Biologiques, 54000 Nancy, France

Abstract—Chemoattractant properties of human thrombin have been studied, by polymorphonuclear leucocyte migration under agarose gel, in the presence of various sulphated macromolecules such as standard heparins, low molecular weight heparins, CY216, K2165, PK10169 and pentosane polysulphate. These compounds did not attract polymorphonuclear leucocytes within the range of concentrations used, whilst thrombin alone is a cytotaxin for these cells. Addition of heparins to thrombin led to an increase in the chemoattractant activity of this enzyme for at least one of the doses studied. Augmentation of the chemoattractant activity of thrombin by heparins was shown at concentrations equivalent to those found in-vivo after administration of therapeutic doses of heparin. Pentosane polysulphate, at the studied concentrations, did not lead to a significant rise in the chemoattractant activity of thrombin.

One of the lesser known properties of heparin is its ability to modify the histological composition of venous thrombi. Thus, in its presence, the thrombi contain a higher number of polymorphonuclear leucocytes (PMNs) than one observes in its absence (Henry 1965; Doutremepuich et al 1985, 1987). It is likely that these cells reach thrombi by being attracted by chemoattractant agents released during coagulation, such as thrombin (Bizios et al 1986; Morin et al 1989). This enzyme's action is not sufficient to explain the greater number of PMNs observed within thrombi generated in the presence of heparin. This observation seems to be explainable only by the anticoagulant's participation in the recruitment of PMNs.

To elicit the possible action of heparin on the chemoattractant property of human thrombin, the chemoattractant activity of thrombin was studied in the presence of various standard heparins (Dakota, Fournier, Roche and Choay), low molecular weight heparins (LMWHs) (CY216, K2165 and PK10169) and pentosane polysulphate.

Materials and methods

The chemoattractant activity of thrombin with or without the compounds tested, was determined under sterilized conditions.

Materials. Chemotaxis was studied using the following compounds: Dextran T 500 (Pharmacia, Sweden) at 2% in (mM) 154 NaCl, 163 NH_4Cl, pH 7·4; glucose phosphate saline buffer (glucose PBS) ((mM) 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1·4 KH_2PO_4, 10 glucose); agarose (indubiose A37, IBF, France); concentrated minimum essential medium (MEM) (Gibco, UK); heat-inactivated human serum stored at −80°C; 893 mM $NaHCO_3$ aqueous solution (Gibco, UK), methanol (Coopérative Pharmaceutique Française, France); formaldehyde (Prolabo, France); 1% ethanolic basic fuschine (Coopérative Pharmaceutique Française, France); 10^{-7} M *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (Sigma, France).

Human thrombin at 3050 NIH units mg^{-1} was purchased from Sigma (France) (A grade, mol. wt = 39 kDa).

The sulphated macromolecules tested were: standard heparins from Dakota (25 000 int. units mL^{-1} batch 88K15, Dakota, France), Fournier (25 000 int. units mL^{-1}, batch F112, Fournier, France), Roche (5000 int. units mL^{-1}, batch F025Q, Roche, France) and Choay (5000 int. units mL^{-1}, batch 1812, Choay, France); low molecular weight heparins CY216 (25 000 units anti-XaIC mL^{-1}, batch 40201, Fraxiparine Choay, France), K2165 (25 000 int. units anti-Xa mL^{-1}, batch 4663401, Fragmine, Kabi Vitrum, France) and PK10169 (100 mg mL^{-1}, batch 062, Lovenox Pharmuka, France); and pentosane polysulphate (100 mg mL^{-1}, batch 50, Hémoclar Clin Midy, France). All were diluted in MEM with added 1% $NaHCO_3$.

Isolation of leucocytes. Venous blood from healthy donors was collected on 110 mM citrate (1 vol for 9 vol blood), cleared from platelets by centrifugation (230 *g*, 20°C, 15 min) and mixed with dextran. A sedimentation of 30 min was allowed to collect a supernatant rich in leucocytes. Red blood cells were lysed by mixing 1 vol supernatant with 4 vol cold NH_4Cl for 10 min. After centrifugation (230 *g*, 20°C, 8 min) the leucocyte pellet was washed with glucose PBS and then resuspended in the same buffer. The number of cells was adjusted to 2×10^7 PMNs mL^{-1}.

Chemotactic migration under agarose gel. The technique used was as described by Nelson et al (1975) and Marchand Arvier & Vigneron (1982).

In an agarose medium, pairs of wells, a and b (separated by 3 mm), were cut. Well a received the substance to be tested, whilst well b was filled with leucocyte suspension.

For both positive and negative controls well b was filled with the leucocyte suspension; well a was filled with the chemoattractant FMLP for the positive control and was empty for the negative control.

An incubation of 3 h at 37°C in humidified atmosphere with 5% CO_2 allowed the migration of PMNs. The reaction was stopped by the addition of methanol (30 min) and formaldehyde (30 min). Cells were then stained with fuschine.

Chemotactic mobilities and spontaneous mobilities were measured for each pair of wells under a microscope and their ratio expressed as the chemotactic index (CI).

Tested substances. Human thrombin solution ($2·8 \times 10^{-5}$ M) in MEM was mixed with an equivalent volume of standard heparin, LMWH or pentosane polysulphate. Mixtures were incubated for 30 min at 37°C with shaking.

In all preparations the thrombin final concentration was of $1·40 \times 10^{-5}$ M, a concentration where thrombin has a maximal chemotactic activity vs PMNs in our experimental conditions (Morin et al 1989).

The final concentrations of heparins and pentosane polysulphate were as indicated in Tables 1 and 2, and were chosen because they correspond to levels found in-vivo after administration during therapy.

Analysis of results. The results were validated using the positive and negative controls. For the positive controls CI < 1·20 demonstrates a chemotactic dysfunction and the corresponding results were rejected. Only negative control CI values ⩽ 1·0 were accepted.

As the action of the compounds tested on the chemoattractant activity of thrombin were determined during separate experiments, we arbitrarily attributed a value of 100% to the mean CI

Correspondence: A. Morin, Centre Régional de Transfusion Sanguine et d'Hématologie, 54511 Vandoeuvre Les Nancy Cedex, France.

Table 1. Action of different concentrations of standard heparins on the chemoattractant activity of thrombin. Results (mean ± s.e.m.) are expressed as percentage of chemoattractant activity of a thrombin-heparin mixture with respect to thrombin alone.

Heparin	Heparin concn (int. units mL^{-1})			
	0·083	0·166	0·332	2·78
Dakota	117·8 ± 15·3*	111·4 ± 7·18*	111·7 ± 11·7*	108·0 ± 11·4*
Fournier	112·0 ± 14·2*	110·6 ± 12·5*	111·6 ± 19·3*	94·7 ± 10·6
Roche	114·1 ± 16·9*	110·3 ± 19·6	109·6 ± 18·4	105·2 ± 22·0
Choay	104·8 ± 10·0	107·4 ± 10·5	110·8 ± 9·95*	103·5 ± 12·9

* $P < 0·05$ compared with thrombin alone.

obtained for thrombin alone (CI = 1·30 ± 0·19). We then determined the percentage of chemoattractant activity corresponding to the mean CI taken for thrombin mixed with a given dose of the compound studied.

Fifteen CI values were collected for each substance. Statistical analysis was carried out using Student's *t*-test. $P < 0·05$ was considered to be significant.

Results

Chemoattractant activity of heparins. In the absence of thrombin, at all concentrations used, the different sulphated macromolecules tested showed no chemoattractant activity. The CI values obtained were 0·91 ± 0·09.

Influence of standard heparins on the chemattractant activity of human thrombin. Standard heparins led to a significant increase ($P < 0·05$) in the chemoattractant activity of thrombin at all concentrations studied for Dakota calcium heparin, at the three lower concentrations used for Fournier calcium heparin, at the lowest concentration for Roche sodium heparin and at the third concentration used for Choay sodium heparin (Table 1).

Influence of LMWHs on the chemoattractant activity of human thrombin. LMWHs led to a significant rise ($P < 0·05$) in the chemoattractant activity of thrombin for the first three concentrations tested of CY216 and K2165, and at 0·980 and 1·96 μg mL^{-1} PK10169 (Table 2).

Influence of pentosane polysulphate on the chemoattractant activity of thrombin. Statistical analysis revealed that pentosane polysulphate produced no significant increase in the chemoattractant activity of thrombin (Table 2).

Discussion

The aim of this work was to reveal a possible influence of heparin on the already known chemoattractant properties of human thrombin for PMNs (Bizios et al 1986; Morin et al 1989). In order to show whether the chemoattractant activity of thrombin may be modified by heparin, we tested four standard heparins and three LMWHs.

The results showed that the studied heparins led to a significant increase in the chemoattractant activity of thrombin. This took place in at least one of the four doses of heparin tested and especially at concentrations that could be found in-vivo after administration during therapy. However, some variations in the responses were observed from one preparation to another. This can probably be explained by the large diversity in composition between the preparations.

The results seem to indicate that calcium heparins (Dakota and Fournier heparins, CY216) act on thrombin more strongly than sodium heparins (Roche and Choay heparins, K2165, PK10169).

The chemotactic response to the thrombin-heparin mixture does not seem to be influenced by the molecular size. LMWHs which are composed of small molecules (2000–8000 Da) are as efficient as standard heparins (up to 30 000 Da).

The anti-IIa and anti-Xa activities of the heparin preparations tested do not seem to have an influence on the chemoattractant behaviour of the heparin-thrombin mixture. Each preparation has its own variation of anti-IIa and anti-Xa activities, because LMWHs have a comparatively weak anti-IIa activity compared with standard heparins. The inverse was obtained for anti-Xa activity.

The mechanism by which heparin increased the attractive property of thrombin was not revealed in this work. However,

Table 2. Action of different concentrations of sulphated macromolecules (LMWHs and pentosane polysulphate) on the chemoattractant activity of thrombin. Results (mean ± s.e.m.) were expressed as percentage of chemoattractant activity of thrombin-sulphated macromolecule mixture with respect to thrombin alone.

Sulphated macromolecule	Sulphated macromolecule concn			
CY216 LMWH	129·4 ± 19·0*	125·9 ± 23·4*	115·3 ± 18·3*	106·0 ± 15·2
(units anti-XaIC mL^{-1})	0·178	0·356	0·712	5·95
K2165 LMWH	111·6 ± 14·7*	112·3 ± 12·3*	122·7 ± 16·2*	103·9 ± 14·1
(int. units anti-Xa mL^{-1})	0·073	0·146	0·292	2·44
PK10169 LMWH	102·6 ± 6·20	106·2 ± 6·00*	110·7 ± 9·18*	101·5 ± 6·10
(μg mL^{-1})	0·490	0·980	1·96	16·0
Pentosane polysulphate	104·1 ± 9·99	108·3 ± 12·4	105·0 ± 9·78	102·2 ± 12·5
(μg mL^{-1})	0·490	0·980	1·96	16·0

* $P < 0·05$ compared with thrombin alone.

other authors have shown that when a given heparin is put in contact with thrombin, interactions take place between these two molecules. The thrombin-heparin association is non-specific, electrostatic and seems to result in a conformational change of the thrombin molecule (Smith 1977; Nordenman & Bjork 1977, 1978; Oshima 1989).

This molecule possesses a particular structural domain which is responsible for its chemoattractant activity (Morin et al 1990). It is possible that the conformational change brought about by the binding of heparin makes the chemoattractant domain of this enzyme more accessible, thus increasing its attractive activity.

The heparin-thrombin interaction does not take place on a specific site of the heparin molecule contrary to the heparin-antithrombin III interaction (Bjork & Lindahl 1982; Choay et al 1983). This interaction being electrostatic, it could take place with various sulphated macromolecules. To check whether sulphated macromolecules other than heparin can modify the chemoattractant activity of thrombin, we studied the action of pentosane polysulphate. Pentosane polysulphate is a sulphated polymer of D-xylose and methyl D-glucuronic acid. Within the same range of concentrations as heparin this compound did not modify, in any significant way, the chemoattractant activity of thrombin, probably due to the difference in sugar composition between heparin and pentosane polysulphate.

References

Bizios, R., Lai, L., Fenton, J. W., Malik, A. B. (1986) Thrombin induced chemotaxis and aggregation of neutrophils. J. Cell Physiol. 128: 485–490

Bjork, I., Lindahl, U. (1982) Mechanism of the anticoagulant action of heparin. Mol. Cell Biochem. 48: 161–182

Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B., Gatti, G. (1983) Structure activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti factor Xa activity. Biochem. Biophys. Res. Commun. 116: 492–499

Doutremepuich, C., Kuttler, M. C., Bousquet, F., Quilichini, R., De Mascarel, M., Toulemonde, F., Vairel, E. (1985) Etude histologique d'une thrombose veineuse induite chez le rat. Ann. Pharm. Fr. 43: 161–164

Doutremepuich, C., Toulemonde, F., De Seze, O., Pereira, F., Anne, M. C., Doutremepuich, F. (1987) Influence of heparin and low molecular weight heparin fraction of the leukocytes in an experimental venous thrombosis model. J. Leukocyte Biol. 42: 628–631

Henry, R. L. (1965) Leukocytes and thrombosis. Thromb. Diath. Haemorrh. 13: 35–46

Marchand Arvier, M., Vigneron, C. (1982) Le chimiotactisme des polynucléaires neutrophiles: moyens d'étude. Feuillet de Biologie 23: 31–33

Morin, A., Marchand Arvier, M., Doutremepuich, F., Vigneron, C. (1989) Coagulation impact on chemotactic activity generation for polymorphonuclear leukocytes. Thromb. Res. 59: 979–984

Morin, A., Marchand Arvier, M., Doutremepuich, F., Vigneron, C. (1990) Localization of the structural domain responsible for the chemotactic properties of thrombin on polymorphonuclear leukocytes. Thromb. Res. 60: 33–42

Nelson, R. D., Quie, P. G., Simmons, R. L. (1975) Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115: 1650–1656

Nordenman, B., Bjork, I. (1977) Purification of thrombin by affinity chromatography on immobilized heparin. Thromb. Res. 11: 799–808

Nordenman, B., Bjork, I. (1978) Studies of the binding of heparin to prothrombin and thrombin activity. Thromb. Res. 12: 755–765

Oshima, G. (1989) Time dependent conformational change of thrombin molecules induced by sulfated polysaccharides. Chem. Pharm. Bull. 37: 1324–1328

Smith, G. F. (1977) The heparin thrombin complex in the mechanism of thrombin inactivation by heparin. Biochem. Biophys. Res. Commun. 77: 111–117