Biochemical Pharmacology, Vol. 38, No. 11, pp. 1865–1867, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

Toxicity of polymorphonuclear neutrophils against hepatocytes: protective effect of heparin

(*Received* 25 *August*, 1988; *accepted* 2 *January* 1989)

Heparin is a highly anionic and sulfated molecule [1] that has been found to interact with many proteins. *In vitro*, it has been shown to bind to and to inhibit lysosomal enzymes [2] including some neutral proteinases of polymorphonuclear neutrophils (PMN)* [1, 3]. Recently, we have demonstrated that stimulated PMN were toxic to isolated rat hepatocytes, and that this toxicity was mediated by proteinases released from PMN [4]. The aim of this study was therefore to investigate the effect of heparin on the toxicity of PMN against hepatocytes.

Materials and methods

The Primaria 24-well plates and the RPMI 1640 medium were obtained from Falcon (Div. of Becton Dickinson, Oxnard, CA) and Gibco (Uxbridge, U.K.), respectively. Glutamine and foetal calf serum were purchased from Flow Laboratories (Irvine, U.K.). Sigma Chemical Co. (St Louis, MO, U.S.A.) supplied bovine serum albumin, zymosan, α_1-proteinase inhibitor, soybean trypsin inhibitor and heparin (from porcine intestinal mucosa). The 4-methyl-umbelliferyl conjugates were obtained from Koch-Light Laboratories (Colnbrook, U.K.).

Effect of heparin on the cytotoxicity of PMN against hepatocytes. Hepatocytes were isolated by collagenase digestion from male Sprague–Dawley rats [5]. Cell viability, estimated by a high membrane refractivity under light microscope [5], was similar in all experiments and was 97 ± 1%. PMN were obtained from the citrated venous blood of healthy volunteers according to Dallegri *et al.* [6]. PMN-mediated cytotoxicity against hepatocytes was quantified by the release of alanine aminotransferase activity (ALT, EC 2.6.1.2) from hepatocytes, a reliable test of hepatocyte alterations [4]. Briefly, 1×10^5 hepatocytes were seeded into flat-bottomed wells of Primaria 24-well plates. After plating, hepatocytes were incubated in the presence of 2×10^6 PMN in a final volume of 400 μl RPMI 1640 containing 2 mM glutamine, 100 I.U./ml penicillin, 100 μg/ml streptomycin and 5 mg/ml bovine serum albumin (culture medium). The toxicity of PMN was studied in basal conditions or after stimulation with 1 mg/ml zymosan opsonized with fresh human serum, as previously described [7]. After a 18 hr incubation at 37° in the presence of 5% CO_2, an aliquot of the culture supernatant was removed to determine the ALT activity released by hepatocytes and cytotoxicity was expressed as the percentage of ALT activity released [4]. The effect of heparin on PMN-mediated toxicity was tested after addition of heparin to the hepatocyte monolayer, 30 min before the addition of PMN. Concentrations studied ranged from 2 to 50 μg/ml.

Effect of stimulated PMN on hepatocytes preincubated with heparin. In these experiments, hepatocytes were preincubated for 6 hr with or without heparin, and then washed before the addition of PMN. Such a 6 hr preincubation time was chosen because it has been shown to allow an optimum binding of heparin to hepatocyte plasma membrane [8]. Cytotoxicity against hepatocytes was determined after a further 18 hr incubation with PMN stimulated by opsonized zymosan (OZ), as described above.

Effect of heparin on the cytotoxicity of PMN lysate and granule proteins against hepatocytes. The PMN lysate was prepared by freezing and thawing 25×10^6 cells in one ml culture medium. The lysate was then diluted 1/1 with culture medium. A crude PMN granule pellet was prepared according to Connelly *et al.* [9]. Granules were disrupted by freezing, thawing and sonication, and then diluted with culture medium. Four hundred microlitres of either lysate or granule preparation were added to 1×10^5 hepatocytes, in the absence or in the presence of heparin. Cytotoxicity was quantified by the release of ALT activity in a 18 hr assay.

Effect of heparin on PMN stimulation. PMN were suspended at a concentration of 5×10^6 cells/ml in Hanks balanced salt solution, pH 7.4, and preincubated 30 min in the absence or in the presence of heparin. Control buffer or OZ were then added and PMN were incubated during an additional 1 hr at 37°. At the end of the incubation period, the tubes were centrifuged at 400 *g* for 10 min and the supernatant was decanted. The activities of *N*-acetyl-β-glucosaminidase (EC 3.2.1.30) and β-glucuronidase (EC 3.2.1.31) released in the supernatant were measured by the fluorometric method of Peters *et al.* [10] using 4-methyl-umbelliferyl conjugates. The determination of *N*-acetyl-β-glucosaminidase was performed at pH 5.8 and that of β-glucuronidase at pH 5.0. At these pH, it was checked that heparin did not inhibit these enzyme activities. The results were expressed as the percentage of the total activity obtained by lysing PMN with 0.1% Triton X 100 (v/v).

Statistical analysis. Results were analysed with a matched-pairs test adapted to small samples [11]. Results were expressed as mean ± 1 SE.

Results and discussion

As shown in Fig. 1, OZ-stimulated PMN induced a 38.0 ± 5.9% ALT release from hepatocytes, and this tox-

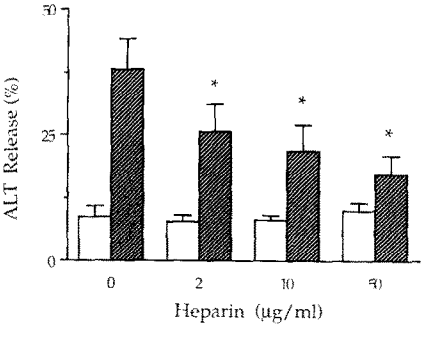

Fig. 1. Effects of heparin on the toxicity of PMN against hepatocytes. Each reaction mixture contained 2×10^6 unstimulated (opened bars) or opsonized zymosan-stimulated (hatched bars) PMN, 1×10^5 hepatocytes and varied concentrations of heparin. The toxicity of PMN against hepatocytes was studied after a 18 hr incubation and was expressed as the percentage of ALT activity released from hepatocytes. Results are expressed as mean ± 1 SE of 4 experiments. * $P < 0.05$ when compared to hepatocytes incubated without heparin.

* Abbreviations used: PMN, polymorphonuclear neutrophils; ALT, alanine aminotransferase activity; OZ, opsonized zymosan.

icity was markedly reduced by heparin. Indeed, the toxicity was decreased by 32% ($P < 0.05$) at a concentration as low as 2 μg/ml and by 55% ($P < 0.05$) at 50 μg/ml. By contrast, unstimulated PMN exhibited mild toxic effects (8.7 ± 2.3% ALT release) that were not significantly modified by heparin (Fig. 1). At the concentrations used in this study, heparin alone had no toxic effect to hepatocytes. In addition, it did not interfere with the determination of ALT activity.

We have previously shown that the toxicity of OZ-stimulated PMN against hepatocytes was mediated by the release of proteinases from stimulated PMN [4]. The effect of heparin that we observed in the present work could theoretically be due either to an inhibition of PMN degranulation or to a protection of hepatocytes against the toxicity of PMN-secreted proteinases. The first hypothesis seems unlikely, since concentrations of heparin ranging from 2 to 50 μg/ml did not significantly reduce the release of two PMN lysosomal enzymes, i.e. *N*-acetyl-β-glucosaminidase and β-glucuronidase (Fig. 2). This result contrasts with that of Brestel and McClain [12], who showed that heparin was able to reduce by nearly 50% the release of myeloperoxidase (another lysosomal enzyme) from OZ-stimulated PMN. The apparent discrepancy between the two studies might be related to the use of a 20-fold higher concentration of heparin by Brestel and McClain [12]. The second hypothesis, i.e. a protection of hepatocytes against the toxicity of proteinases, is favored by the results of experiments using PMN lysate as a source of neutrophil proteinases. As shown in Fig. 3, PMN lysate induced a 23.7 ± 3.5% ALT release that was significantly decreased by about 60% by 2, 10 and 50 μg/ml heparin ($P < 0.02$). It was checked that the toxicity of PMN lysate, as that of intact stimulated PMN [4], was essentially mediated by proteinases. Indeed, α_1-proteinase inhibitor, soybean trypsin inhibitor as well as foetal calf serum, which contains naturally occurring antiproteinases, decreased the ALT release by 70, 76 and 46%, respectively (Fig. 3). Moreover, toxicity to hepatocytes was also observed after addition of a crude preparation of PMN granule proteins (25 μg/well), and again this toxicity was reduced by heparin.

The mechanism by which heparin protects hepatocytes

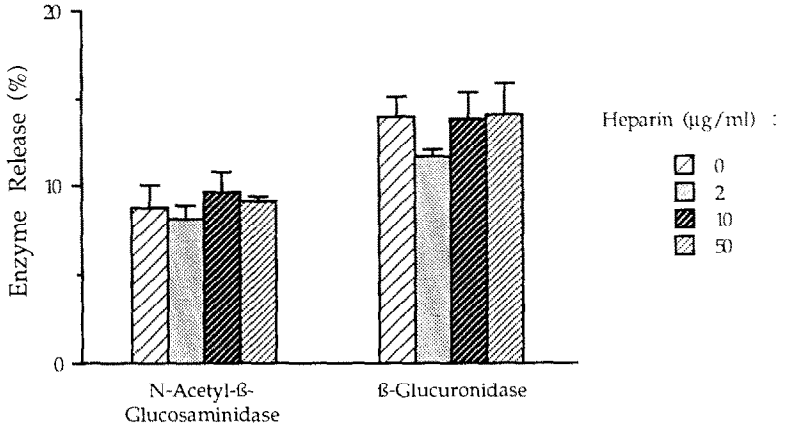

Fig. 2. Effects of heparin on the release of *N*-acetyl-β-glucosaminidase and β-glucuronidase from stimulated PMN. After a 30 min preincubation with varied concentrations of heparin, PMN were incubated with 1 mg/ml opsonized zymosan for 1 hr. The concentrations of *N*-acetyl-β-glucosaminidase and β-glucuronidase present in the cell-free supernatant were measured according to the method described in Materials and Methods. Results were expressed as the percentage of the total activity obtained by lysing PMN with 0.1% Triton X 100 (v/v) (mean ± 1 SE of 4 experiments).

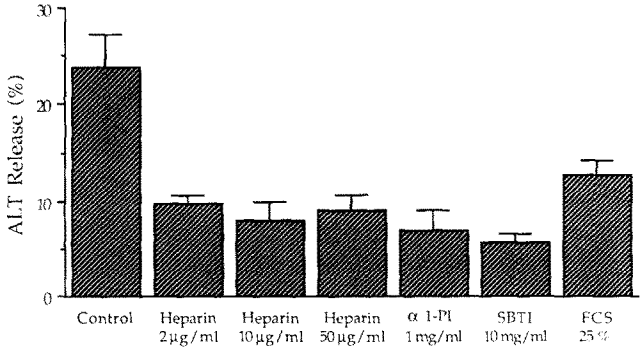

Fig. 3. Effects of heparin on the toxicity of PMN lysate against hepatocytes. The PMN lysate was prepared by freezing and thawing 25×10^6 cells/ml. The lysate was added to 1×10^5 hepatocytes that were incubated for 18 hr in the absence or in the presence of varied concentrations of heparin, α_1-proteinase inhibitor (α_1-PI), soybean trypsin inhibitor (SBTI) or foetal calf serum (FCS). The toxicity of lysate against hepatocytes was expressed as the percentage of ALT activity released from hepatocytes. Results are expressed as mean ± 1 SE of 4 experiments.

is not known. One explanation could be that heparin inhibits the toxicity of stimulated PMN by its binding to receptors present on hepatocyte plasma membrane [8]. This explanation seems unlikely since we observed that, when hepatocytes were preincubated with heparin, and then washed before the addition of PMN, they were not protected from PMN toxicity (45.2 ± 2.9% ALT release after preincubation without heparin vs 42.0 ± 7.1% after preincubation with 50 μg/ml heparin). The likely explanation is that the protective effect of heparin is due to its binding to proteinases released by stimulated PMN. Actually, it is well established that heparin is able to bind to and to inactivate proteinases released from human PMN, e.g. chymotrypsin- and elastase-like enzymes [1, 3].

PMN are known to play a major role in acute inflammatory reaction [13], and it has been suggested that heparin, besides its anticoagulant effect, had anti-inflammatory properties [12]. It is tempting to speculate that this effect of heparin might be in part explained by the inactivation of mediators released by PMN.

In summary, the effect of heparin was studied on the proteinase-mediated toxicity of human PMN against isolated rat hepatocytes. Opsonized zymosan-stimulated PMN were markedly toxic to hepatocytes and this cytotoxicity was inhibited by 32 to 55% by concentrations of heparin ranging from 2 μg/ml to 50 μg/ml. This effect was not due to an inhibition of the stimulation of PMN since heparin did not decrease the release of two neutrophil lysosomal enzymes, *N*-acetyl-β-glucosaminidase and β-glucuronidase. It was explained by the inhibition of the post-secretory step of PMN toxicity since heparin reduced by 60% the toxicity of a proteinase-containing PMN lysate and by 69% that of a preparation of PMN granule proteins.

Acknowledgements—We are greatly indebted to Ms L. Rosario for typing the manuscript.

INSERM U-99
Département de Pathologie Tissulaire et Cellulaire
Hôpital Henri Mondor
94010 Creteil
France

PHILIPPE MAVIER
ANNE-MARIE PREAUX
JEAN ROSENBAUM
ELIE SERGE ZAFRANI
DANIEL DHUMEAUX

REFERENCES

1. Marossy K, Interaction of the chymotrypsin- and elastase-like enzymes of the human granulocyte with glycosaminoglycans. *Biochim Biophys Acta* **659**: 351–361, 1981.
2. Avila JL and Convit J, Physicochemical characteristics of the glycosaminoglycan-lysosomal enzyme interaction *in vitro*. *Biochem J* **160**: 129–136, 1976.
3. Redini F, Tixier J-M, Petitou M, Choay J, Robert L and Hornebeck W, Inhibition of leucocyte elastase by heparin and its derivatives. *Biochem J* **252**: 515–519, 1988.
4. Mavier P, Préaux A-M, Guigui B, Lescs M-C, Zafrani ES and Dhumeaux D, *In vitro* toxicity of polymorphonuclear neutrophils to rat hepatocytes: Evidence for a proteinase-mediated mechanism. *Hepatology* **8**: 254–258, 1988.
5. Laperche Y, Préaux A-M, Feldmann G, Mahu J-L and Berthelot P, Effect of fasting on organic anion uptake by isolated rat liver cells. *Hepatology* **1**: 617–621, 1981.
6. Dallegri F, Frumento G and Patrone F, Mechanisms of tumour cell destruction by PMA-activated human neutrophils. *Immunology* **48**: 273–279, 1983.
7. Harlan JM, Killen PD, Harker LA, Stricker GE and Wright DG, Neutrophil-mediated endothelial injury *in vitro*. Mechanisms of cell detachment. *J Clin Invest* **68**: 1394–1403, 1981.
8. Kjellén L, Oldberg A, Rubin K and Höök M, Binding of heparin and heparan sulphate to rat liver cells. *Biochem Biophys Res Commun* **74**: 126–133, 1977.
9. Connelly JC, Skidgel RA, Schulz WW, Johnson AR and Erdös EG, Neutral endopeptidase 24.11 in human neutrophils: Cleavage of chemotactic peptide. *Proc Natl Acad Sci USA* **82**: 8737–8741, 1985.
10. Peters TJ, Muller M and DeDuve C, Lysosomes of the arterial wall. I Isolation and subcellular fractionation of cells from normal rabbit aorta. *J Exp Med* **136**: 1117–1134, 1972.
11. Schwartz D, *Méthodes Statistiques à l'Usage des Médecins et des Biologistes*. Flammarion Médecine Sciences, Paris, 1969.
12. Brestel EP and McClain EJ, A mechanism for inhibition of luminol-dependent neutrophil chemiluminescence by polyanions. *J Immunol* **131**: 2515–2519, 1983.
13. Wilhem DL, Inflammation and healing. In: *Pathology* (Eds. Anderson WAD and Kissane JM), pp. 25–89. CV Mosby Company, Saint-Louis, 1977.

Biochemical Pharmacology, Vol. 38, No. 11, pp. 1867–1869, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

Metabolism of diethylnitrosamine by microsomes of human respiratory nasal mucosa and liver

(*Received* 12 *October* 1988; *accepted* 2 *January* 1989)

Although the incidence of nasal tumors in the human population is low, certain individuals such as the workers in the leather or wood industry experience a higher risk of nasal cancer [1].

Nitrosamines can cause tumors in different tissues including the nasal cavity of experimental rodents [2–4]. They may also have a role in inducing cancer in the human nose as well as other respiratory tracts. The volatile diethylnitrosamine (DEN) present in air, water, foods and tobacco smoke [5] can be inhaled or absorbed from other tissues and readily passed to the nose. In rodents DEN, in addition to other nitrosamines administered i.p., reaches the nose in a few minutes [4] and is metabolically activated via the cytochrome P-450 dependent monooxygenases system [4, 6, 7] in the nasal mucosa.

We have recently described the presence of drug-metabolizing enzymes in human respiratory nasal mucosa [8]. In the present study we have investigated whether DEN can be deethylated by human nasal mucosa microsomal enzymes. The results have been compared with those obtained with microsomal preparations from human liver.