Uptake of Fractionated ^{3}H-Heparin by Isolated Rat Kupffer Cells

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Purpose and Methods. The uptake of fractionated ^{3}H-heparin by isolated rat Kupffer cells was examined to determine the uptake mechanism. ***Results.*** The association of fractionated ^{3}H-heparin was concentration-dependent with a dissociation constant of 3.4 nM and a maximum association capacity of 1.3 pmol/10^6 cells, suggesting the involvement of a specialized mechanism. Although 2,4-dinitrophenol inhibited neither the association nor internalization of fractionated ^{3}H-heparin, lowering the temperature from 37°C to 4°C reduced the internalization of fractionated ^{3}H-heparin by 70% without affecting the association. ***Conclusions.*** It is suggested that the uptake mechanism may differ from receptor-mediated endocytosis of polypeptides and be mediated by scavenger receptors, because organic anions, and several ligands of scavenger receptors, as well as several heparin analogs, inhibit the binding of fractionated ^{3}H-heparin to Kupffer cells, while phenylarsine oxide, which is known to inhibit the receptor-mediated or absorptive endocytosis of polypeptides, inhibits neither the association nor internalization of fractionated ^{3}H-heparin.

KEY WORDS: fractionated ^{3}H-heparin; Kupffer cells; scavenger receptor; endocytosis.

INTRODUCTION

Heparin, a water-soluble mucopolysaccharide, is widely used as an anticoagulant (1). Recently, newly found biological activities, such as its anti-human immunodeficiency virus effect (2), have attracted interest because of their clinical applications. However, heparin is polydisperse with regard to its molecular weight and heterogenous as far as its biological and chemical properties are concerned. The polydispersity and heterogeneity can be disadvantages for heparin therapy, including its disposition in the body. We, therefore, fractionated commercial heparin to obtain heparin with a defined biological activity and molecular weight.

Our previous studies in rats showed that the fractionated heparin is more rapidly eliminated from plasma and accumulated to a greater extent by a liver, compared with unfractionated heparin (3,4), and taken up by both parenchymal and non-parenchymal cells in a dose-dependent manner after intravenous administration (5). In parenchymal cells in primary culture, a specialized uptake mechanism has been suggested to be involved in the uptake (6–8). However, the uptake mechanism in non-parenchymal cells, another subpopulation of liver cells, has scarcely been investigated.

In this study, the effects of concentration, temperature, and various compounds on the uptake of fractionated ^{3}H-heparin were investigated in isolated rat Kupffer cells to determine the uptake mechanism of fractionated ^{3}H-heparin.

MATERIALS AND METHODS

Materials

[^{3}H(G)]Heparin sodium salt of porcine mucosal origin (#2643-177; 130.0 U/mg and 16 MBq/mg) was purchased from DuPont-NEN Co. (Boston, Mass.). Heparin sodium salt of porcine mucosal origin (#29F-0314; 178 U/mg) was obtained from Sigma Chemical Co. (St. Louis, Mo.). RPMI1640 medium and uncoated plastic dishes were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan) and Sumitomo Bakelite Co. (Tokyo, Japan), respectively.

Tritiated and unlabeled heparin were fractionated as described in our previous reports (3,4) to obtain a heparin fraction with affinity for protamine-Sepharose and a molecular weight of 20,000 Da.

Isolation of Kupffer Cells

Non-parenchymal cells were isolated from non-fasting male Wistar rats, weighing 180–230 g (6 to 7 weeks old), as described previously (5), and Kupffer cells were purified from the non-parenchymal cells by the method of D. Fiete et al. (9). Non-parenchymal cells were identified by their diameter of about 10 μm, and less than 10% contaminated with parenchymal cells. Non-parenchymal cells (more than 95% viable by the trypan blue exclusion test) were seeded in uncoated plastic dishes (2 ml/dish) at a density of 6.5×10^5 cells/ml in RPMI1640 medium and maintained in 95% air—5% CO_2 at 37°C for 15 min. The dishes were then washed thoroughly twice with 2 ml RPMI 1640 medium to obtain Kupffer cells, about 75% of the total non-parenchymal cells attached to the uncoated plastic dishes (10). The procedure of purifying Kupffer cells by letting them attach to the uncoated plastic dishes has been widely used, and verified by peroxidase and phagocytic activity (10–12).

Uptake and Binding Experiments

Test solutions were prepared in Hanks' basic salt solution supplemented with 10 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) and adjusted to pH 7.4; the solutions contained 2.5, 5, 10, 25 and 50 nM fractionated ^{3}H-heparin. For the two highest concentrations, unlabeled fractionated heparin was added to achieve the desired concentrations. Kupffer cells, which were attached to the plastic dishes, were washed twice with 2 ml Hanks' basic salt solution containing HEPES (pH 7.4). Uptake was initiated by adding 2 ml of test solution (pH 7.4) to each dish with shaking at 50 strokes/min and 37°C, and was stopped by rapid removal of the test solution at specified times and washing the cells twice with ice-cold Hanks' basic salt solution. Kupffer cells were dissolved in 1 ml 3N NaOH for one hour at 37°C and neutralized with 1 ml 3N HCl. The samples were then mixed with 5 ml Scintisol EX-H (Dojindo Co., Kumamoto, Japan) to determine the radioactivity. Protein content was determined by the method of Lowry et al. (13) at the end of each experiment and converted to cell numbers

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using the relationship of 6.7×10^6 cells/mg protein, which was determined in advance.

Internalized fractionated ^{3}H-heparin was estimated as undissociated fractionated ^{3}H-heparin with an excess amount (500 nM) of unlabelled fractionated heparin for 30 min at 4°C. The binding was measured as the uptake at 4°C for 60 min in the presence of the appropriate compound.

To examine the effects of various inhibitors on the uptake of fractionated ^{3}H-heparin, after preincubation with inhibitor for 20 min at 37°C, the cells were incubated with 5 nM fractionated ^{3}H-heparin at 37°C for 3 h in 95% air—5% CO_2 in the presence of inhibitor. When examining the effect of 2,4-dinitrophenol, only the effect of pretreatment was examined by measuring the uptake in the absence of inhibitor. Phenylarsine oxide was dissolved in dimetylsulfoxide and diluted 500-fold in test solution.

The equation, Association = $B_{max} \cdot C/(K_d + C)$, was fitted to the equilibrium association at 60 min versus the concentration (C) profile to estimate the maximum association capacity (B_{max}) and dissociation constant (K_d) using a nonlinear regression program, MULTI (14).

RESULTS AND DISCUSSION

Concentration-Dependent Association

The time course of the association of fractionated ^{3}H-heparin in isolated rat Kupffer cells was found to reach equilibrium by 60 min, as shown in Fig. 1. The association of fractionated ^{3}H-heparin was concentration-dependent with a maximum association capacity (B_{max}) of 1.3 pmol/10^6 cells and a dissociation constant (K_d) of 3.4 nM, suggesting the involvement of a specialized mechanism in Kupffer cells (Fig. 2). The values of B_{max} and K_d were one and two orders of magnitude, respectively, smaller than those in cultured parenchymal hepatocytes (6), a K_d of 89 nM and B_{max} of 118 pmol/10^6 cells, suggesting that fractionated ^{3}H-heparin is involved in a mechanism with a higher affinity and lower capacity for isolated rat Kupffer cells than that in cultured parenchymal hepatocytes. The lower dissociation constant in Kupffer cells may partly explain our previous observation *in vivo* that the contribution of Kupffer cells to the hepatic uptake of fractionated ^{3}H-heparin is larger at lower doses (5).

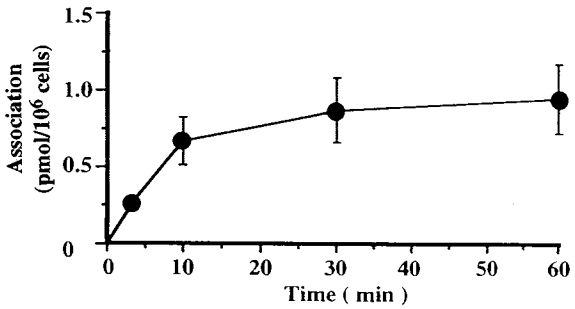

Fig. 1. Time-course of the association of fractionated ^{3}H-heparin in rat Kupffer cells. Each point and vertical bar represents the mean ± S.D. for three rats. The association was measured at 37°C and a concentration of 5 nM fractionated ^{3}H-heparin.

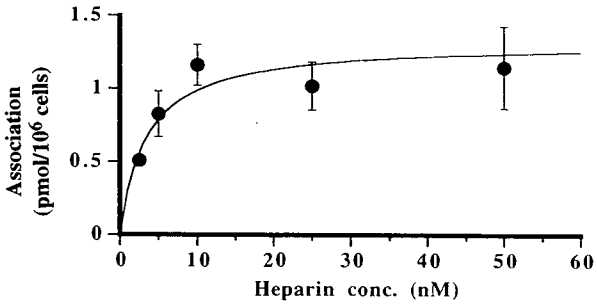

Fig. 2. Concentration-dependent association of fractionated ^{3}H-heparin in rat Kupffer cells. Each point and vertical bar represents the mean ± S.D. for three rats. The solid line represents the computer-fitted profile. The association was measured at 37°C and 60 min.

Effects of Temperature and Transport Inhibitors on the Uptake

The internalized fractionated ^{3}H-heparin, which was estimated as undissociated fractionated ^{3}H-heparin after washing with an excess amount (500 nM) of unlabeled fractionated heparin at 4°C, was about 15% of the association under the control conditions as shown in Fig. 3. Although the amount internalized was not very large, fluid-phase endocytosis as a non-specific uptake mechanism for macromolecules with a clearance reported to be 1.6 μl/day/10^6 cells (15), can explain less than 1% of the internalization of fractionated ^{3}H-heparin. 2,4-Dinitrophenol, a metabolic inhibitor, inhibited neither the association nor the internalization of fractionated ^{3}H-heparin, indicating that both binding and internalization of fractionated ^{3}H-heparin in Kupffer cells are ATP-independent. However, lowering the temperature from 37° to 4°C reduced the internalization of fractionated ^{3}H-heparin by 70% without affecting the association.

In addition, phenylarsine oxide, which has been reported to inhibit the internalization in the receptor-mediated and absorptive endocytosis of peptides (16–18), inhibited neither the association nor the internalization of fractionated ^{3}H-heparin; neither did chloroquine, an inhibitor of the endosomal degradation and receptor recycling (19), nor cycloheximide, an inhibitor of the protein synthesis (8). These results suggest that the specialized uptake of fractionated ^{3}H-heparin into rat Kupffer cells may differ from the receptor-mediated or absorptive endocytosis of polypeptides.

Although a small amount of fractionated ^{3}H-heparin, 5% of the association, appeared to be internalized at 4°C in Fig. 3, it was comparable with the uptake measured in the presence of 500 nM unlabeled fractionated heparin (data not shown). This may represent the non-specific binding of fractionated ^{3}H-heparin to the cell membrane, and, hence, the internalization of fractionated ^{3}H-heparin may be completely inhibited at 4°C.

Effects of Various Compounds on Binding

To further characterize the specialized uptake mechanism of fractionated ^{3}H-heparin, the effects of various compounds on the binding of fractionated ^{3}H-heparin were investigated. As shown in Fig. 4, the binding of fractionated ^{3}H-heparin to isolated Kupffer cells was inhibited by several

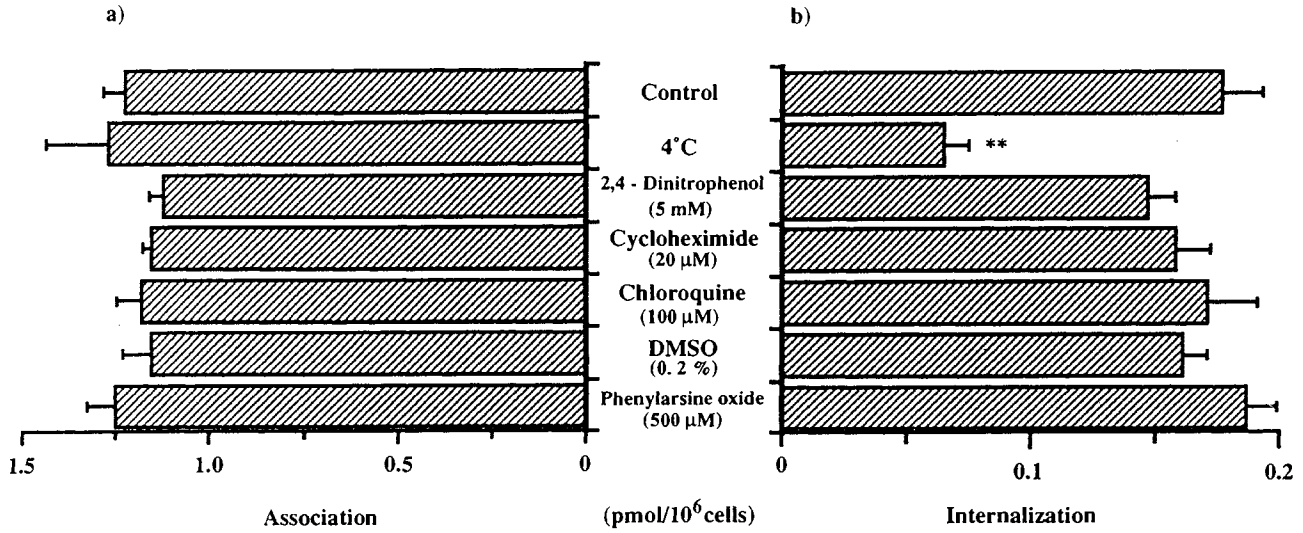

Fig. 3. Effects of temperature and transport inhibitors on the association (a) and internalization (b) of fractionated ^{3}H-heparin in rat Kupffer cells. Each column and vertical bar represents the mean ± S.D. for three rats. After the preincubation of Kupffer cells in the presence of a transport inhibitor for 20 min, the association and internalization were measured at 37°C, and a concentration of 5 nM fractionated ^{3}H-heparin at 3 h. * Significantly different from the control value at $p < 0.05$.

heparin analogs (heparan sulfate, chondroitin sulfate and pentosan polysulfate), organic anions (rose bengal and sulfobromophthalein), and 4,4′-diisothiocyanatostilben-2,2′disulfonic acid (DIDS), an inhibitor of the anion transport system (20), suggesting that a negative charge and/or heparin-like structure may play an important role in the binding of fractionated ^{3}H-heparin to Kupffer cells. N-Acetylgalactosamine and mannose, ligands of the galactose and mannose receptor (21), respectively, did not inhibit binding.

The ligands of the scavenger receptors (dextran sulfate, fucoidan and polyguanylic acid (22,23)) inhibited the binding of fractionated ^{3}H-heparin, suggesting the involvement of scavenger receptors in the binding of fractionated ^{3}H-heparin. However, polycytidylic acid did not affect the binding of fractionated ^{3}H-heparin. Polycytidylic acid, which is an analog of polyguanylic acid, cannot bind to scavenger receptors due to the difference in steric-configuration compared with polyguanylic acid (24). The selective inhibition by polyguanylic acid provides an additional evidence for the involvement of scavenger receptors in the binding of fractionated ^{3}H-heparin. Furthermore, the dissociation constant of 3.4 nM for fractionated ^{3}H-heparin is close to that reported for maleylated bovine serum albumin (4.8–30 nM) and acetylated low density lipoprotein (2.3–6.5 nM) in terms of their binding to purified rat liver scavenger receptor proteins (25). The scavenger receptors have recently been suggested to be involved in the uptake of heparin in the rat *in vivo* (26) and in macrophage-like cells, RAW 264.7 (23). The uptake of low molecular weight heparin was reduced by coadministration of maleylated bovine serum albumin in the

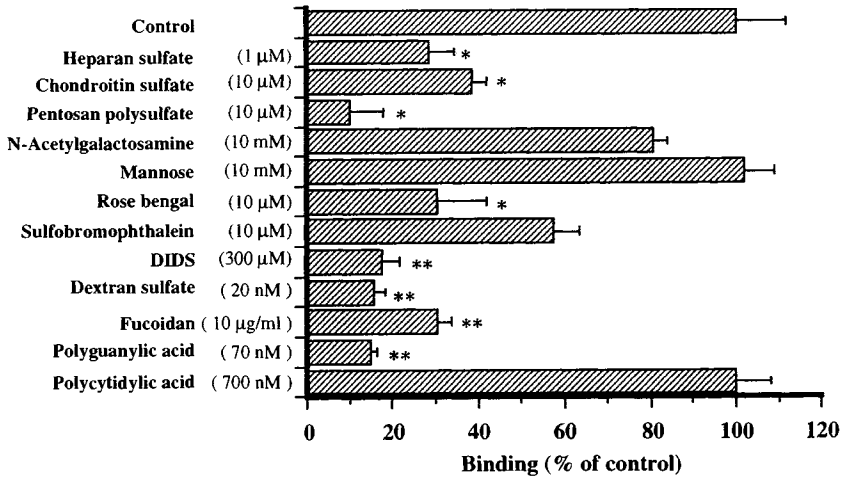

Fig. 4. Effects of various compounds on the binding of fractionated ^{3}H-heparin to rat Kupffer cells. Each column and vertical bar represents the mean ± S.D. for three rats. The binding was measured at 4°C, and a concentration of 5 nM of fractionated ^{3}H-heparin at 60 min. The control value was 0.95 pmol/10^6 cells. Significantly different from the control value at $p < 0.05$ (*) and $p < 0.01$ (**).

former case and the binding of unfractionated heparin was inhibited by fucoidan in the latter case. The present study is the first to suggest the involvement of scavenger receptors in the uptake of heparin in Kupffer cells based on the inhibitory effects of ligands of the scavenger receptors.

In conclusion, the present study shows that the association of fractionated ^{3}H-heparin in isolated rat Kupffer cells is concentration-dependent and lowering the temperature from 37°C to 4°C inhibits the internalization of fractionated ^{3}H-heparin without affecting association. The binding of fractionated ^{3}H-heparin is inhibited by several ligands of scavenger receptors, while phenylarsine oxide, which is known to inhibit the receptor-mediated or absorptive endocytosis of polypeptides, inhibits neither the association nor internalization of fractionated ^{3}H-heparin. These results suggest that the endocytosis mediated by scavenger receptors, which is different from the receptor-mediated or absorptive endocytosis of polypeptides, may be involved in the uptake of fractionated ^{3}H-heparin by Kupffer cells.

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