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ASSOCIATION OF GALACTOSAMINE-INDUCED HEPATITIS IN THE RAT WITH HYPERHYALURONANAEMIA AND DECREASED HYALURONAN UPTAKE BY THE ISOLATED, PERFUSED LIVER

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Abstract—Plasma hyaluronan (HA) concentration and the rate of HA uptake by the isolated, perfused liver were measured in rats treated with saline, D-galactosamine (GalI-NH_2, 50 mg/100 g body wt), gadolinium chloride ($GdCl_3$) (0.5 mg/100 g body wt), and $GdCl_3$ + GalI-NH_2. $GdCl_3$ was given 24 hr before GalI-NH_2 or saline. Plasma L-alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2), a marker for hepatocyte damage, was increased by 8 hr and remained elevated for 24 hr after GalI-NH_2 injection. $GdCl_3$ did not affect plasma enzyme levels when given alone or in association with, but prior to, GalI-NH_2. Plasma HA levels were increased (200%) within 2 hr after GalI-NH_2 administration. A plateau was reached at 8 hr, which was maintained for at least 24 hr. Although $GdCl_3$ alone did not affect plasma HA levels, it slightly delayed the increase in HA concentration in GalI-NH_2-treated rats. Livers, isolated 24 hr after GalI-NH_2 treatment, exhibited a severe depression (approximately 67%) of HA uptake. $GdCl_3$ did not prevent this suppression. The data presented indicate that: (1) one of the sinusoidal endothelial cell-dependent functions of the liver, i.e. removal of HA from the blood stream, is profoundly impaired during galactosamine-induced hepatitis, and (2) the adverse effect of GalI-NH_2 on this sinusoidal endothelial cell function may not be dependent on $GdCl_3$-suppressible Kupffer cell functions.

Extensive experimental work on the mechanisms underlying the hepatotoxicity of D-galactosamine (GalI-NH_2†) has established that the main event leading to hepatitis is a transient depletion of the uridine nucleotide pool within hepatocytes, causing a severe deficit in the synthesis of macromolecules (for a review see Ref. 1). More recent experimental work has shown that another event should be considered as contributory to the development of GalI-NH_2 hepatitis: namely, the activation of Kupffer cells which leads to secretion of mediators that can damage the hepatocyte [2, 3]. Secretion of mediators is also present in hepatic injury produced by Gram-negative bacterial lipopolysaccharides (LPS), whose effects are potentiated by GalI-NH_2 [4, 5]. LPS is a powerful stimulator of Kupffer cells [6], and most of the deleterious effects of LPS on the liver are mediated by Kupffer cell activation. Therefore, it appears likely that GalI-NH_2-induced hepatotoxicity also involves, at least in part, a component of intercellular communication within the liver.

Within the context of intercellular communication in the liver, the impact of Kupffer cells on hepatocyte functions is relatively well studied (see Ref. 7 for a review). However, less information is available on their influence on sinusoidal endothelial cells (SEC). This is due, in part, to a lack of adequate procedures to assess the functional state of these cells. Such a lack was overcome by an important discovery in the field of liver SEC biology [8, 9], namely that these cells have the property to take up hyaluronan (HA), a widely spread, blood-borne glycosaminoglycan, through a process of receptor-mediated endocytosis. Further work [10] has established that Kupffer cells also possess HA endocytotic receptors and are capable of HA uptake. However, the quantitative contribution of Kupffer cells to HA uptake by the whole liver is at least one order of magnitude lower than that of SEC [10, 11]. Such a difference allows HA uptake by the liver to be considered primarily a result of SEC activity. In a previous study [11], we characterized HA uptake by the isolated, perfused rat liver and showed that it can be taken as an index of SEC functional state. In the present study we use this model: (1) to assess the effect of GalI-NH_2 administration on liver SEC function as reflected by blood HA concentration and HA uptake by the isolated, perfused rat liver, and (2) to determine whether Kupffer cells mediate GalI-NH_2 effects on liver SEC.

Our results demonstrated that: (1) GalI-NH_2-induced hepatitis is associated with a marked increase in blood HA concentration and a marked decrease

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† Abbreviations: AOA, alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2); GalI-NH_2, 2-amino-2-deoxy-D-(+)-galactose; HA, hyaluronic acid (hyaluronan); LPS, lipopolysaccharide(s); and SEC, sinusoidal endothelial cell(s).

in hepatic HA uptake, and (2) inactivation of the large Kupffer cell population by $GdCl_3$ does not abolish the effects of Gal-NH_2.

MATERIALS AND METHODS

The experiments reported in this study were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86–23, 1985).

Treatment of animals. Male, Sprague–Dawley rats (320–380 g; Charles River, Wilmington, MA) were used 6 days after their arrival in our animal care facility. During this period they were provided food and water *ad lib*. On day 1 of the experiment, at around 9:00 a.m., rats were anesthetized with Rompun® (xylasine) and Ketaset® (ketamine) (0.9 and 9 mg/100 g body wt, intramuscularly) to implant two PE50 catheters intravascularly (jugular vein and carotid artery). Both catheters were exteriorized. Immediately after surgery, animals received $GdCl_3$ ($GdCl_3 \cdot 6H_2O$, 0.72 mg/100 g body wt, in sterile saline, 3.6 mg·mL^{-1}), or an equivalent volume of sterile saline (control), by a slow (3–4 min) intravenous injection. After surgery, animals had free access to food and water. The following day, at approximately 9:00 a.m., animals received either Gal-NH_2 (D-(+)-galactosamine·HCl, Sigma Chemical Co., St. Louis, MO, 50 mg/100 g body wt, intraperitoneally, in sterile saline, adjusted to pH 6–7 with NaOH), or sterile saline (control). When a second dose of $GdCl_3$ was employed, it was given to animals intravenously just prior to the Gal-NH_2 injection. For the next 24 hr the food was removed but animals had free access to water. Serial, arterial blood samples (300 μL) were obtained on citrate (5%, w/v, 10 μL/100 μL blood). Sampled blood was replaced by injecting an equivalent amount of sterile saline. Plasma was obtained by centrifugation at 13,000 *g* for 2 min, and used for the assay of HA and alanine:2-oxoglutarate aminotransferase (AOA, EC 2.6.1.2) activity. Animals were euthanized 24 hr after Gal-NH_2 administration to perform liver perfusion experiments. A separate series of experiments was performed to measure HA uptake by the perfused livers 2 hr after Gal-NH_2 administration.

Liver perfusion. This was performed in a non-recirculating system using hemoglobin-free, Krebs–Ringer bicarbonate buffer that was continuously gassed with O_2:CO_2 (95:5%) in a rotary-disc oxygenator [12], at 36.5°, and a flow rate of 38–40 mL·min^{-1}. Twenty minutes after portal vein cannulation, taken as zero time of the perfusion, effluent samples were collected at 2-min intervals with the aid of a peristaltic pump and a fraction collector. HA was infused into the portal cannula for 20 min, through an in-line infusion chamber with the aid of computerized infusion pumps (Type 22, Harvard Apparatus, South Natick, MA), at a rate of 1.8 mL·min^{-1}, to give a final concentration of 150–180 ng·mL^{-1}. This concentration has been determined in a previous study [11] to be saturating with respect to the rate of HA uptake for the M_r of HA used (1.3×10^6 Da; prepared from rooster comb; Sigma Chemical Co.). Influent perfusate samples were collected at 4-min intervals. Other details of the liver perfusion procedure have been published elsewhere [13].

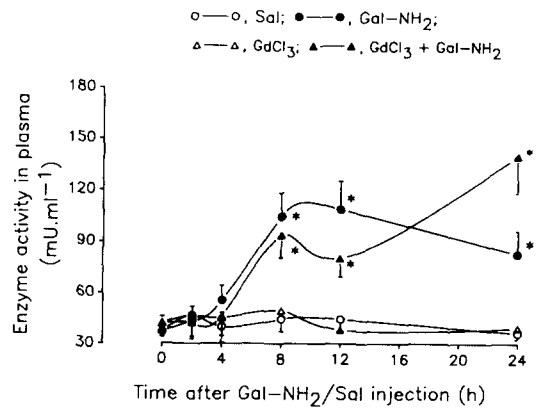

Fig. 1. L-Alanine:2-oxoglutarate aminotransferase activity in plasma of saline-, Gal-NH_2-, $GdCl_3$-, and $GdCl_3$ + Gal-NH_2-treated rats. Values are means ± SEM for N = 4 in each group. Asterisks indicate a statistically significant difference ($P < 0.05$) as follows: Gal-NH_2 vs Sal; $GdCl_3$ + Gal-NH_2 vs $GdCl_3$.

Analytical procedures. HA was quantified in 50 μL of plasma and 100 μL of influent and effluent perfusate samples, using a radiometric procedure (Kabi Pharmacia Diagnostics, Inc., Piscataway, NJ). HA uptake was calculated from the difference in HA concentration between the influent and effluent perfusate, corrected for the flow rate and liver weight. The activity of AOA was measured in 50 μL of plasma, using a commercial kit (Sigma Chemical Co., Cat. No. 59-10).

Statistical analysis. A two-way ANOVA repeated measure analysis was performed on the treatment groups over time. Significant interaction was encountered for each of the three parameters measured. Therefore, a one-way repeated measure analysis was performed for each group independently over time. Time-dependent differences were determined by the Student–Newman–Keuls test. Additionally, each time interval was tested independently by one-way ANOVA, and significant differences between groups were determined by the Student–Newman–Keuls test. Differences at a P equal to or smaller than 0.05 were considered statistically significant.

RESULTS

Serum AOA activity, a marker of parenchymal cell cytolysis, was low and remained virtually unchanged in saline- and $GdCl_3$-injected rats during the 24-hr experimental period (Fig. 1). In Gal-NH_2-treated rats, the enzyme activity increased significantly by 8 hr (104.6 ± 23.8 vs 44.5 ± 7.3 mU·mL^{-1} in control, $P < 0.05$), and remained elevated through the 24-hr observation period. This effect of Gal-NH_2 was not prevented by previous administration of $GdCl_3$ (Fig. 1).

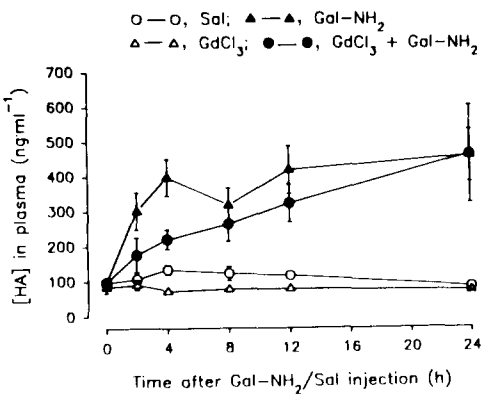

Fig. 2. Plasma hyaluronan concentration ([HA]) as a function of time in saline-, Gal-NH_2-, $GdCl_3$-, and $GdCl_3$ + Gal-NH_2-treated rats. Values are means ± SEM for N = 4 in each group. The following statistics apply. *As a function of time*: in Gal-NH_2 and $GdCl_3$ + Gal-NH_2 groups the time points between 2 and 24 hr did not differ from each other, but were different ($P < 0.05$) from their corresponding zero time values. In Sal- and $GdCl_3$-treated groups, no statistically significant, time-dependent changes were seen. *As a function of treatment:* no difference was observed between the groups at zero time; a difference at $P < 0.05$ was seen between Gal-NH_2- and Sal-treated groups, and between $GdCl_3$ + Gal-NH_2 and $GdCl_3$-treated groups; the only significant difference ($P < 0.05$) between Gal-NH_2 and $GdCl_3$ + Gal-NH_2 groups was seen at 2- and 4-hr time points.

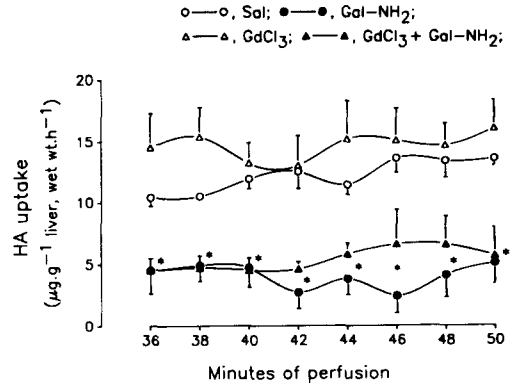

Fig. 3. Hyaluronan (HA) uptake by the perfused livers, isolated 24 hr after saline-, Gal-NH_2-, $GdCl_3$-, and $GdCl_3$ + Gal-NH_2-treatment. Values are means ± SEM for N = 4 in each group. Asterisks indicate a statistically significant difference ($P < 0.05$) between Gal-NH_2 vs Sal, and $GdCl_3$ + Gal-NH_2 vs $GdCl_3$ groups. No statistically significant difference was found between Gal-NH_2- and $GdCl_3$ + Gal-NH_2-treated rats.

As shown in Fig. 2, serum HA concentration in saline-injected animals, which at zero time was $96.9 \pm 9.8\ ng \cdot mL^{-1}$ (N = 4), did not change significantly over the 24 hr experimental period; nor did it change after $GdCl_3$ administration to rats. In contrast, HA concentration was elevated significantly 2 hr after Gal-NH_2 administration ($299.9 \pm 54.2\ ng \cdot mL^{-1}$, N = 3, $P < 0.01$ vs time-matched control), when it virtually reached a plateau. In $GdCl_3$ + Gal-NH_2-treated rats, plasma HA levels also increased significantly compared with saline or $GdCl_3$ treatment alone. However, the kinetics were slightly different from what was seen in Gal-NH_2-treated rats, in that the increase was delayed. Thus, 2 hr after Gal-NH_2 injection, although slightly increased, plasma HA concentration was significantly lower than in the Gal-NH_2-treated group. A plateau was reached only 4 hr after Gal-NH_2 administration and was virtually the same as in the group treated with Gal-NH_2 alone. No measurements were performed after the 24 hr time point.

Hepatic HA uptake was inhibited severely (67%) by Gal-NH_2 treatment (Fig. 3). The rate of HA uptake ranged between 10.4 and 13.6 $\mu g \cdot$(g liver wet wt)$^{-1} \cdot hr^{-1}$ (with a mean value of 12.1 $\mu g \cdot$(g liver wet wt)$^{-1} \cdot hr^{-1}$) in saline-injected rats and between 2.4 and 5.1 $\mu g \cdot$(g liver wet wt)$^{-1} \cdot hr^{-1}$ (with a mean value of 4.0 $\mu g \cdot$(g liver wet wt)$^{-1} \cdot hr^{-1}$) in Gal-NH_2-treated rats ($P < 0.05$ between the groups). Treatment of animals with $GdCl_3$ alone did not affect hepatic HA uptake, nor did $GdCl_3$ prevent the inhibitory effect of Gal-NH_2.

Administration of two doses of $GdCl_3$ to rats, prior to either saline or Gal-NH_2 injection, did not affect plasma HA concentration or hepatic HA uptake (data not shown). Also, livers isolated 2 hr after Gal-NH_2 administration, i.e. at the time point when an increase in plasma HA levels was already evident, but plasma AOA activity was not yet changed, displayed a diminished capacity (44%) to take up HA (data not shown). However, this inhibition was significantly lower ($P < 0.05$) than the one observed in the livers 24 hr after Gal-NH_2 administration.

DISCUSSION

Plasma HA concentration is determined by the rate of its influx from the interstitium and its clearance from the blood. The latter is accomplished by lymph nodes, spleen and liver [14–19]. It has been established that under *in vivo* conditions, the liver plays a major role in HA extraction from the blood [15–19]. As documented in the introduction, liver HA uptake is predominantly accomplished by SEC, with Kupffer cells having a very small quantitative contribution. Therefore, impaired capacity of liver SEC to take up and degrade HA can result in increased circulating HA levels. This is demonstrated by the fact that a number of liver diseases, including alcoholic hepatitis, cirrhosis of different etiologies, liver injury after ischemia, and others [20–22], are associated with increased plasma HA concentrations. Moreover, the tight correlation between plasma HA levels and severity of various liver diseases makes it possible to evaluate the functional state of liver SEC by measuring plasma HA concentration [21].

In this study we have demonstrated that Gal-NH_2-induced hepatitis is associated with increased plasma HA levels, concomitant with a marked inhibition of

HA uptake by the isolated, perfused rat liver. In view of the role of the liver in HA clearance, it is likely that the inhibition of hepatic HA uptake is, in part, responsible for the increased plasma HA concentration. Furthermore, our data allow us to dissociate between the deleterious effects of GaI-NH_2 on the hepatocyte, on the one hand, and on the SEC, on the other. Our results show that GaI-NH_2-induced lesion(s) of liver SEC, as reflected by their decreased HA uptake capacity, preceded GaI-NH_2-induced hepatocyte damage, that is the increase in plasma HA levels occurred at an earlier time point (2 hr) after GaI-NH_2 administration than the increase in serum AOA activity (4 hr). The latter is taken as an index of enhanced parenchymal cell cytolysis. Moreover, the early (2 hr) increase in plasma HA level was concomitant with a 44% inhibition of hepatic HA uptake (data not shown).

The mechanism(s) underlying the suppression of hepatic HA uptake by *in vivo* GaI-NH_2 treatment is not known. It has become increasingly apparent that GaI-NH_2 induced-hepatitis is mediated not only by the transient depletion of the uridine nucleotide pool, but also by extrahepatic factors. Among the latter, Gram-negative bacterial LPS has been ascribed a central role, since: (a) its blood concentration increases after GaI-NH_2 administration [23–25]; (b) LPS is a potent Kupffer cell activator, leading to secretion of various mediators [6], and (c) suppression of Kupffer cell activity by administration of various agents, e.g. methyl-palmitate, alleviates the symptoms of GaI-NH_2-induced hepatitis [26]. Some of the mediators secreted by Kupffer cells (i.e. superoxide anion, nitric oxide and leukotrienes) can be injurious to both the hepatocyte and SEC. The existence of an LPS-induced, Kupffer-cell mediated effect on SEC (specifically, on SEC HA uptake capacity) seems plausible and is supported by previous findings from our laboratory, showing that: (a) LPS administration increases plasma HA levels concomitantly with a marked suppression of hepatic HA uptake [18], and (b) $GdCl_3$ administration prior to LPS treatment abolished these LPS-induced changes*. However, whether the GaI-NH_2-induced changes in hepatic HA uptake are mediated by LPS remains questionable for the reasons given below.

In an attempt to clarify whether the deleterious effects of GaI-NH_2 on hepatic HA uptake are mediated by Kupffer cell activation, we used $GdCl_3$ to suppress Kupffer cell functions. The rationale for using $GdCl_3$ is based on: (a) our previous results, showing that $GdCl_3$ abolishes the inhibitory effect of LPS on hepatic HA uptake in rats*, and (b) the above-cited literature data implicating, at least in part, LPS as a mediator of GaI-NH_2-induced hepatitis. It is known that the effects of i.v. administered $GdCl_3$, in doses similar to the one employed in the present study, are confined to Kupffer cells, leaving other liver cells unaffected [27]. Primarily, these effects consist of phagocytosis suppression in a first phase, followed by elimination of a large Kupffer cell population at a later phase after the drug administration [28]. Therefore, in the first phase, only the phagocytosis-associated processes, such as superoxide anion and eicosanoid secretion, are diminished by $GdCl_3$. Our results show that $GdCl_3$, given in one or two doses, did not prevent the inhibitory effect of GaI-NH_2 on hepatic HA uptake, seen at 24 hr after GaI-NH_2 administration. These findings suggest that $GdCl_3$-suppressible Kupffer cell functions do not participate in the mediation of GaI-NH_2 inhibitory effects on hepatic HA uptake. This conclusion is also supported by data, showing that increased blood LPS levels in GaI-NH_2-treated rats cannot be detected earlier than 3 hr after GaI-NH_2 administration [23], whereas our data show that increased blood HA levels and the attendant inhibition of hepatic HA uptake occurred as early as 2 hr after the drug administration. Therefore, LPS participation in GaI-NH_2-induced suppression of hepatic HA uptake is unlikely at least during the first phase of drug action.

An issue that needs further clarification is $GdCl_3$-induced delay in the increase in plasma HA concentration during the first 4 hr after GaI-NH_2 administration. This may be regarded as a protective action of $GdCl_3$ against the initial deleterious effect of GaI-NH_2 on HA uptake. However, such an action may be exerted at sites other than the liver, because, as documented above, LPS could not be detected in the blood of GaI-NH_2-treated rats earlier than 3 hr after drug administration. Therefore, an LPS-dependent Kupffer cell activation, within 3 hr after GaI-NH_2-administration, is not likely to take place. The possibility that GaI-NH_2 affects HA uptake by sinusoidal endothelial cells at sites other than the liver, modifying their responsiveness to GaI-NH_2, cannot be excluded, but it remains speculative in nature. Experimental work is needed to study in more detail the effect of GaI-NH_2 on SEC functions.

In interpreting our data one has also to consider another function of Kupffer cells, i. e. LPS-induced tumor necrosis factor-α (TNF-α) secretion, which is not altered by $GdCl_3$ administration†. However, TNF-α participation in the regulation of hepatic HA uptake can also be ruled out on the basis of results showing that: (a) treatment of rats with anti-TNF-α IgG prior to LPS administration does not prevent the inhibitory effect of LPS on hepatic HA uptake [18], and (b) addition of TNF-α to cultured rat liver SEC does not affect their capacity to take up and degrade HA [29].

The data presented in this study taken together with our previous results*, and the literature data presented in the foregoing discussion are compatible with the assumption that while LPS effects on SEC are likely to be mediated by Kupffer cells, specifically through their $GdCl_3$-suppressible functions, GaI-NH_2 effects are likely to result from a direct action

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† Iimuro Y, Yamamoto M, Kohno H, Itakura J, Fujii H and Matsumoto Y, Blockade of liver macrophages by gadolinium chloride reduces lethality in endotoxemic rats. Analysis of mechanisms of lethality in endotoxemia. Manuscript submitted for publication. Cited with permission of the authors.

of the drug on SEC. More experimental work is needed to clarify this issue.

Finally, the pathophysiologic significance of increased circulating HA levels is not known. In view of the multiple role played by HA in a number of cellular functions (for a review, see Ref. 30), one can speculate that increased plasma HA concentration may interfere with various aspects of host defense providing, thus, a virtual link between liver disease and immune functions.

In conclusion, our study demonstrated that: (1) an important liver function, essentially linked to SEC, i.e. HA scavenging, is impaired in GaI-NH_2-induced hepatitis, and (2) such an effect may not be mediated by the $GdCl_3$-suppressible functions of Kupffer cells.

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