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Hepatocellular injury inhibits lectin-mediated tumor colonization into BALB/c-mice livers

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Abstract. Acute (hepatitis) and chronic (cirrhosis) liver injuries were experimentally induced in BALB/c-mice by administration of D-galactosamine and carbon tetrachloride, respectively. In both experimental liver diseases the incidence of hepatic tumor colonization of sarcoma L-1 was significantly reduced as compared to non-treated control animals. Thus, it seems that either dysfunction or loss of organ-characteristic lectins (galactosyl-specific hepatic lectins) prevented liver colonization. Histochemical staining of liver sections from D-galactosamine or carbon tetrachloride-treated mice with appropriate galactose-containing (neo)glycoproteins supported this hypothesis, since the lectin-dependent binding was greatly reduced as compared to sections from non-treated animals.
Key words. BALB/c-mice; liver cirrhosis; acute hepatitis; liver lectins; metastasis formation.

After vertebrate lectins had been discovered[1], and their importance in the hepatic clearence of serum asialoglycoproteins had been recognised[2,2], we suggested that those organ-characteristic lectins may also act as adhesion molecules in the metastatic process by interacting with carbohydrate moieties on the surfaces of tumor cells[4,5]. The hepatic lectin (HL) shows a specificity for terminal galactose (Gal) and N-acetylgalactosamine (GalNAc) which are common constituents of membrane glycoproteins[6,7]. Therefore, HLs obviously play an important role in cell-cell interactions on the basis of Gal/GalNAc receptor recognition[8,9].

Recent experiments have demonstrated the possibility of inhibiting the formation of metastases in the liver by blocking organ lectins with receptor-complementary galactoglycoconjugates or galactose [10,11]. In certain liver diseases (cirrhosis, hepatitis, fatty infiltration) a marked increase of serum asialoglycoproteins[3,12] was shown, apparently due to alterations of the hepatocyte plasma membrane and decrease (or loss) or dysfunction of HLs[13]. Accordingly we postulated and proved that the adherence of tumor cells in diseased livers was greatly diminished and that the occurrence of liver metastases was the exception rather than the rule[14].

In the following paper we prove that HL dysfunction (or loss) in certain experimental liver diseases results in a significantly reduced incidence of colony-formation by sarcoma L-1 cells in the liver in BALB/c-mice.

Materials and methods

Chemicals. Phosphate buffered saline (PBS) was obtained from Flow Laboratories GmbH, Meckenheim, Germany; RPMI-1640 from GIBCO, Grand Island, N.Y., USA; D-galactosamine and carbon tetrachloride (CCl_4) from E. Merck, Darmstadt, Germany.

Animals. Inbred male BALB/c-mice (Central Institute for Experimental Animals, Hannover, Germany), 8–12 weeks old, weighing 20–22 g were used throughout the studies. The animals were kept in plastic cages and allowed free access to food and water.

Tumor. For all experiments sarcoma L-1 tumor (Institute of Oncology, Warsaw, Poland) was used. This tumor arose spontaneously in the lung of a BALB/c-mouse and was maintained in this species[15]. Material was used from 112–116 serial passages of the tumor. Primary tumors were dissected from donor mice, disintegrated with scissors and gently passed through a steel sieve. The cells were washed, suspended in RPMI and counted. All cell suspensions injected into mice were >95% viable as assessed by trypan blue dye exclusion and were examined microscopically for signs of aggregation. Suspensions exhibiting any obvious aggreates were discarded since the extent of tumor cell clumping can affect colonization capacity[16]. In order to induce hepatic tumor cell colonization, neuraminidase-treatment of the sarcoma L-1 cells was carried out as previously described[10,11].

Experimental design. In BALB/c-mice ($n = 10$ per experimental group) acute liver cell injury was experimentally induced by intraperitoneal administration of D-galactosamine (0.9 g/kg body weight, 2 days prior to and after tumor cell inoculation) as described elsewhere[13]. A model of liver cirrhosis was established in BALB/c-mice using carbon tetrachloride (2 ml/kg body weight, subcutaneously, twice a week for 8 weeks). The efficacy of the agents was proved with the help of morphological, histological and biochemical parameters as recently described[12,13]. 2 days after final CCl_4 and first D-galactosamine administration a 0.1 ml suspension of 1×10^6/ml RPMI of viable, neuraminidase-treated sarcoma L-1 cells was intravenously inoculated

into the tail veins of BALB/c-mice as described previously[10,11]. Lung and liver tumor nodules were counted 14 days after tumor cell inoculation under a dissecting microscope by two independent observers using the technique of Hill and Bush[17].

Statistics. Student's t-test was used for statistical analysis. All experiments were performed at least twice and yielded reproducible results. For the in vivo studies, experimental groups consisted of 10 BALB/c-mice.

Synthesis of markers for glycocytology. (Neo)glycoproteins containing mannose (Man), fucose (Fuc), galactose (Gal) or lactose (Lac) were provided by Prof. Dr. H.-J. Gabius, Marburg, Germany, and synthesized as previously described[18,19]. Briefly, the diazo or p-isothiocyanato derivatives of p-aminophenyl glycosides, obtained by catalytic hydrogenation of commercially available p-nitrophenyl glycosides (Sigma Chemicals Co., Heidelberg, Germany) were coupled to the carrier molecule bovine serum albumin (BSA; Biomol, Ilvesheim, Germany), which was biotinylated in a further step with a yield of 10 ± 2 carbohydrate moieties per carrier molecule, respectively[20].

Histochemical staining. Histochemical procedures were carried out by routine techniques as described elsewhere[21]. Briefly, liver sections (mounted on glass slides) of non-treated and D-galactosamine or CCl_4-treated BALB/c-mice were rehydrated in graded alcohol (including 30% alcohol containing hydrogen peroxide to block endogenous peroxidase activity). After extensive washing with PBS the sections were incubated in biotinylated BSA (10 µg/ml PBS; this concentration proved to be optimal in preceeding studies) for 30 min at room temperature in high-humidity chambers. Thereafter, sections were rinsed with PBS, incubated with ABC reagent (Camon, Wiesbaden, Germany) and thoroughly washed. Bound biotinylated (neo)glycoproteins were localized in the sections by applying a solution of 0.05% diaminobenzidine in PBS containing 0.015% hydrogen peroxide to the sections for 10 min. Staining was terminated by using distilled water. Control sections were incubated with biotinylated BSA (10–100 µg/ml PBS) and unlabeled (neo)glycoproteins (3-10 µg/ml PBS), gently rinsed with PBS followed by incubation with biotinylated (neo)glycoprotein. The intensity was graded by two independent observers and scored as negative (−), weak (+), medium (++) and strong (+++).

Results

After inducing acute (D-galactosamine hepatitis) or chronic (CCl_4 cirrhosis) liver cell injury in BALB/-c mice (verified patho-histologically at the Institute of Pathology, University of Cologne and biochemically according to Sawamura et al.[12,13]) the challenge with neuraminidase-treated sarcoma L-I tumor cells resulted in a significantly reduced incidence of liver colonies. Compared to a control group of mice, which received intraperitoneal PBS injections, D-galactosamine treatment caused a reduction of hepatic tumor nodules of more than 90%, and carbon tetrachloride treatment even resulted in a complete inhibition of tumor settlement in the liver (table 1). However, the treatment did not influence the homing process elsewhere. The number of tumor colonies in the lung did not differ from that in the control group. Occurrence in additional organs (e.g. in kidney, adrenal gland, spleen or peritoneal cavity) could not be verified. These experimental data are in accordance with recent clinical studies[14,22] which indicated that the development of metastatic liver colonies was greatly reduced in certain liver diseases and obviously due to dysfunction or loss of hepatic lectins.

Biotinylated carrier protein, chemically coupled with Gal-moieties, has been used as histochemical probe for the study of organ-characteristic lectins in murine liver tissue sections. As shown in table 2, a strong binding of Gal-containing (neo)glycoproteins could be verified in liver sections of non-treated BALB/c-mice, indicating the presence of Gal-specific HLs. Preincubation of the liver tissue with unlabeled Gal-containing (neo)glycoprotein inhibited the binding of analogue labeled markers, whereas Man or Fuc-containing (neo)glycoproteins did not interfere with the subsequent Gal-specific binding. This proves the dependence of binding on a structural feature (lectin-carbohydrate interaction), not

Table 1. Mean number of liver and lung colonies ($\bar{x}$) in BALB/c-mice with acute (D-galactosamine hepatitis) and chronic (CCl_4 cirrhosis) liver diseases after challenge with sarcoma L-1 cells.

BALB/c-mice (n = 10 per experimental group)	Sarcoma L-1 cells (1×10^5) neuraminidase-treated	
	$\bar{x}$ liver colonies ($\pm$SD)	$\bar{x}$ lung colonies ($\pm$SD)
PBS-treated (control)	9.3 ($\pm$1.7)	159 ($\pm$17.6)
D-galactosamine-treated	0.3* ($\pm$0.2)	150 ($\pm$28.2)
CCl_4-treated	0*	163 ($\pm$36.5)

SD = standard deviation

* = <0.001, statistically significantly different from control group

Table 2. Staining of murine liver sections with biotinylated (neo) glycoproteins: Gal-galactose, Fuc-fucose, Man-mannose, Lac-lactose. All labeled (neo)glycoproteins were incubated for 30 min at concentrations of 10 µg/ml PBS.

	(Neo)glycoproteins			
	Gal	Fuc	Man	Lac
Normal liver (control)	+++	−	−	++
Normal liver pretreated with				
unlabeled Gal	−	−	−	−
unlabeled Fuc	+++	−	−	++
unlabeled Man	+++	−	−	++
D-galactosamine hepatis	+	−	−	+
CCl_4 cirrhosis	−	−	−	−

− = no staining; + = weak staining/faintly visible; ++ = medium staining; +++ = strong staining.

simple unspecific (charge) interaction. Furthermore, labeled non-glycosylated carrier protein failed to bind under the experimental conditions, excluding a contribution of cell binding by protein-protein interaction. Histological evaluation proved that mainly organ-characteristic parenchymal cells (hepatocytes) within the sections showed evidence of binding sites for the particular sugar. Gal-containing (neo)glycoprotein showed both cytoplasmic and membrane binding to hepatocytes, but no nuclear binding. However, membrane binding was evidently stronger than cytoplasmic binding, suggesting accumulation of binding molecules (lectins) in this area. In experimental liver diseases (D-galactosamine hepatitis; CCl_4 cirrhosis) the binding of Gal-containing carrier protein was markedly reduced or absent in both localizations, cytoplasm and membrane. Thus, severe (experimental) liver diseases result in an evident loss or dysfunction of HLs.

Discussion

Recent experimental data support the hypothesis that adhesion of tumor cells (metastasis formation) and adhesion of bacteria (infectious diseases) have much in common, especially the participation of lectins in this process[23-25]. This lectin-mediated recognition is highly specific. Thus, the hepatocyte β-D-galactose-specific lectins (HLs) recognize (beside the monosaccharide D-galactose) only oligosaccharides with terminal β-linked D-galactose residues which are restricted to certain galactans or galactoglycoconjugates[9-11]. This lectin-mediated receptor interaction is not exclusively related to the number of D-galactose moieties but more to their steric arrangement[2]. In accordance with this postulate, HL-blocking with appropriate complementary glycoconjugates prevented hepatic tumor cell colonization in mice[10,11].

Recently, the development of liver metastases in more than 1500 cancer patients has been reviewed and it was found that in certain liver diseases (cirrhosis, chronic hepatitis, fatty infiltration) the incidence of liver metastases was significantly reduced compared to that in cancer patients with otherwise normal liver[14]. These data were confirmed by Uetsuji et al.[22] who found in 250 patients treated in their surgical department that colorectal cancer metastasizes into the normal liver, but not into the injured liver, especially the cirrhotic liver. We postulated that this reduction of metastatic organ colonization was the result of a reduced function or quantity of liver specific lectins (HL). Experimental data reported here support this hypothesis and show that in acute (hepatitis) and chronic (cirrhosis) liver diseases the incidence of tumor cell colonization in the liver is significantly reduced. Recent reports about decrease (or total loss) of HLs and accumulation of serum asialoglycoproteins in experimental liver diseases such as D-galactosamine hepatitis and CCl_4[3,12,13] cirrhosis suggested that reduced liver colonization may be the result of hepatocyte membrane alterations.

Histochemical studies with Gal-containing (neo)glycoproteins proved that in experimental D-galactosamine hepatitis and CCl_4 cirrhosis the specific lectin-mediated adhesion of labeled marker molecules was greatly diminished or missing. This is obviously evidenced by a loss or dysfunction of organ-characteristic HLs in certain liver diseases, leading to high serum levels of asialoglycoproteins and reduced metastasis formation[13,14].

Thus, HL blocking with receptor analogues as well as HL dysfunction or loss in certain liver diseases can inhibit tumor colonization in this organ. The experimental evidence presented here supports the hypothesis that organ-characteristic lectins play an important role in the organ-specific distribution of metastases.

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