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Malignant transformation in hepatocytes is associated with the general increase of glycoprotein ligands specifically binding to the endogenous lectin CSL*

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ABSTRACT

Several hepatoma cell lines and hepatic ascite tumour cells were studied for the presence of glycoprotein ligands of an endogenous lectin, the "Cerebellar Soluble Lectin" (CSL). This lectin is also present in hepatocytes *in vivo* and *in vitro* and can be detected biochemically and immunologically. In transformed cells, the level of CSL glycoprotein ligands is increased 50-fold as compared to the control cells. Such an increase is not observed for the ligands of the plant lectin, concanavalin A, which is, as CSL, a D-mannose-binding lectin. These results indicated that the changes in glycans during malignant transformation, in these cells, is specifically important for minor glycans binding to CSL.

INTRODUCTION

The molecular basis of cell transformation and malignancy is an important question which remains to be understood. The role played by cell surface glycans in malignant transformation indicates promising clues in this regard. Studies have indicated that contact inhibition of cell growth can be restored by affecting surface glycans. Similarly, it is well documented that surface-glycan composition of tumour cells is considerably modified relative to normal cells (for review, see refs. 1 and 2). The implication of cell-adhesion glycans or tumour-specific glycolipids in transformation has been suggested[1–7]. However, nothing is clear about the nature of the relationship between cell surface carbohydrate modifications and cell transformation. It remains to be known whether changes of surface carbohydrates are the initial event leading to malignant transformation of if they are an epiphenomenon. Endogenous lectins (carbohydrate-binding proteins) have been shown to be modified in various tumour cells[8,9]. The potential roles of these endogenous lectins in modulation of cell contacts in malignant transformation has been hypothesized[10–12]. The isolation[13] of a cerebellar soluble lectin (CSL) and the demonstration that it is involved in the mechanism of cell adhesion–recognition between cells[14–18] afford new possibilities to address the mecha-

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nisms of cell transformation. Evidence for such a concept is derived from the observation that CSL is capable of modulating (increase) cell proliferation upon specific adhesion of oligodendrocytes[15]. Furthermore, it is relevant that some endogenous glycoprotein ligands of CSL in the nervous tissue[14,18] are molecules involved in cell adhesion and possessing HNK-1-like epitopes[19–24]. These carbohydrate epitopes may be found also on transformed cells[3,25,26]. The preliminary observations that a D-mannose-binding lectin, analogous to CSL, was also present in the liver[27] opened new research perspectives in exploring the precise role of this molecule in the liver. We report herein that transformed liver cells have, in contrast to normal hepatocytes, a considerably higher amount and number of endogenous glycoprotein ligands for lectin CSL.

EXPERIMENTAL

Materials. — Horseradish peroxidase (HRP, fraction VI), bovine serum albumin (BSA), and 4-chloro-1-naphthol were obtained from Sigma Chemical Co. (St. Louis, MO, USA); nitrocellulose filters (0.22-μm pore size) from Millipore (Molsheim, France); HRP-conjugated goat anti-rabbit antibodies from Pasteur Diagnostic (Marnes-la-Coquette, France); Bolton and Hunter reagent and Hyperfilm MP from Amersham (Amersham, UK); Ultroser G and concanavalin A (Con A) from IBF (Villeneuve-la Garenne, France); and minimal essential medium Eagle (MEM), Dulbecco's modified Eagle's medium (DMEM), and foetal calf serum from Gibco (Paisley, Scotland).

Normal and transformed liver cells. — HTC cells, highly dedifferentiated, were derived from a Morris hepatoma 7288C (ref. 28) and FAZA cells[29] from the Reuter H35 hepatoma cell line[30]. FAZA cells and HTC cells were routinely grown as monolayers in DMEM supplemented with 10% foetal calf serum, D-glucose (4 g/L), and antibiotics (100 units/mL of penicillin and 1 mg/mL of streptomycin). The ascitic Zajdela hepatoma (strain D) derived from an experimental tumour in male Wistar rats[31] was perpetuated in syngenic animals by intraperitoneal transplantation of 20×10^6 tumour cells. The peritoneal ascitic fluid was punctured eight days later, diluted in buffered saline solution, and cleaned of blood cells by successive low-speed centrifugations (5 min at 60*g*). The adaptation of these ascitic cells to monolayer growth led to the established cell line referred to as Zajdela hepatoma culture[32] (ZHC) cells. They were routinely grown in DMEM medium supplemented with 1% calf serum D-glucose (8 g/L), and antibiotics as just described[32].

Adult hepatocytes were isolated from 2-month-old Wistar rats by perfusing the liver with a collagenase solution[33] and cultured as described earlier[34]. Cell viability exceeded 95% as judged by the Trypan Blue exclusion test. Parenchymal cells (4×10^6) were plated on fibronectin-covered plastic flasks (10 μg/25 cm^2) into MEM H16 medium supplemented with 10% inactivated foetal calf serum and 2% serum substitute Ultroser G (5 mL). The experiments were performed on cells cultured for 48 h.

All cultures were maintained at 37° in a 5% CO_2 atmosphere. For analysis, cells were harvested and washed twice in phosphate-buffered saline solution. The pellet was

directly dissolved in 1% sodium dodecyl sulfate at a concentration of 2 mg/mL of protein[35]. Before electrophoresis, samples were diluted with the Laemmli[36] dissociating buffer (1 vol.) and heated for 5 min at 90°.

Preparation of CSL and liver D-*mannose-binding proteins.* — Isolation of CSL from young rat cerebella was performed by immunoaffinity chromatography as previously described[17,18]. The pure and active lectin, isolated from young rat cerebella, was iodinated with the Bolton and Hunter reagent[37] as modified by Kuchler *et al.*[14] Liver, D-mannose-binding lectins were purified by affinity chromatography on HRP-Sepharose columns as described elsewhere[27].

Electrophoretic techniques. — Poly(acrylamide) gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed in the buffer system of Laemmli[36] using 13% poly(acrylamide) gel slabs (0.75-mm thick). Proteins were stained with Coomassie Brilliant Blue R (CBB). For detection of glycoproteins (as for immunoblotting detection of CSL), proteins were transferred[38] to nitrocellulose filters (0.22-μm pore size), with the difference that 0.04% SDS was included in the blotting buffer. After saturation with periodate-treated BSA[39] (in order to abolish possible artifactual binding of lectins to glycans shared by contaminating globulins in BSA preparations), the filters were incubated either with the Con A–HRP technique[40] (peroxidase was revealed with 4-chloro-1-naphthol[41]) or with iodinated CSL[14]. Blanks were obtained by incubating and washing in the presence of 0.3M D-mannose. Autoradiography of blots was performed with Hyperfilm MP. Immunoblotting detection of CSL was performed as previously described[16].

Immunocytochemical techniques. — Rats were anaesthetized with ether and perfused for 30 min through the portal vein with a mixture of a solution containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.1% picric acid in phosphate-buffered saline (PBS, 15mM Na phosphate buffer containing 150mM NaCl) (100 mL per rat). The liver was dissected and soaked overnight at 4° in the same fixative. Sections (100-μm thick) were obtained with a Lancer vibratome.

Cultures of normal mouse or rat hepatocytes were fixed for 1 h at room temperature in the same fixative. After several rinses in PBS, sections and cultures were incubated with a 1:20 dilution of normal goat serum, followed by 4 h at room temperature with a 1:200 dilution of anti-CSL rabbit antibodies (or pre-immune rabbit serum for controls). After several rinses in PBS, samples were incubated overnight at 4° with a 1:200 dilution in PBS of HRP-labelled, goat-anti-rabbit IgG. After exhaustive washings, sections were revealed with the 3,3'-diaminobenzidine method[42]. Samples for electron microscopy were postfixed in a 2% glutaraldehyde solution in PBS for 1 h at 4°, and then with 1% osmium tetraoxide solution in 0.1M cacodylate buffer, pH 7.2. The sections were dehydrated in ascending ethanol concentrations and embedded in Spurr resin, whereas cultures were embedded in Araldite. Ultrathin sections were obtained with a Reichert microtome and observed with a Philips EM 420 electron microscope.

RESULTS

Presence of lectin CSL in hepatocytes. — As shown in Fig. 1 in the light microscope, CSL was detected immunocytochemically in rat hepatocytes, in cultures (Fig. 1), and *in vivo* (not shown). It seemed to be absent in macrophages and in endothelial cells (not shown). The lectin showed intracellular distribution, particularly concentrated in small dots. The cytoplasm was only poorly stained. In contrast, some staining was observed in the regions of contact between hepatocytes, which suggested the presence of CSL in the junctions between parenchymal cells. The localization was verified by electron microscopy (Fig. 2, a–d) *in vivo* in the rat liver. The intracellular organelles had a shape similar to lysosomes. CSL lectin was particularly concentrated in very short regions of the hepatocyte membrane. It corresponded to areas of contact between hepatocytes, probably tight junctions. This localization suggested that CSL is a molecule involved in the maintenance of the contact between hepatocytes.

As shown in Fig. 3 (lane 3), several lectins could be isolated from rat liver by affinity chromatography on HRP-Sepharose columns and specific elution with 0.3M D-mannose. Several bands displayed M_r values identical with that of purified CSL (Fig. 3, lane 4), and which were significantly different from those of the Ca^{2+}-dependent, D-mannose-binding protein, MBP[43,44].

Glycoprotein ligands of CSL in normal and tumour cells. — As shown in Fig 4a, cultured tumour cells (Fig. 4a, lanes 2–6) possess protein compositions significantly different from those of normal cultured hepatocytes (Fig. 4a, lane 1). When endogenous ligands of CSL were revealed after electrotransfer and detected with iodinated CSL, followed by autoradiography, an enormous difference in the distribution pattern between normal and transformed cells was observed (Fig. 4c). In normal cells (Fig. 4c, lane 1), only one minor glycoprotein ligand of CSL (M_r 110 kDa) could be detected in very small amount. This differed totally from the situation in transformed cells (Fig. 4c, lanes 2–6) where a very intense labelling was found associated with a very high number of glycoprotein bands. The binding of iodinated CSL is believed to be specific since it did

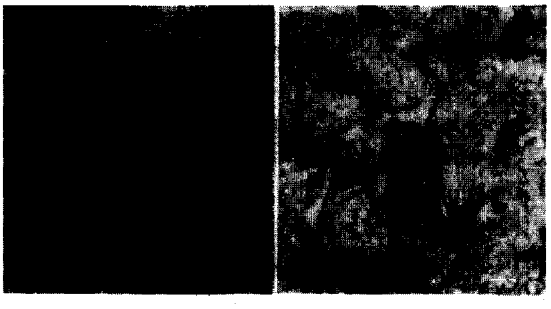

Fig. 1. Immunoperoxidase demonstration of the presence of lectin CSL in cultures of normal rat hepatocytes by light microscopy. Magnification, × 750. Note the presence of intracellular intensively-stained small granules in positive samples (A) in contrast to controls (B), obtained by use of prei-mmune rabbit serum instead of specific anti-CSL antibodies.

Fig. 2. Immunoperoxidase localization of CSL in rat liver with the electron microscope. Bars represent 0.3 μm. Anti-CSL immunoreactivity was found in some intracellular organelles (B) having the size of lysosomes (LYS; empty arrows). Very light staining was observed in multivesicular bodies (MVB). Mitochondria (M) were totally unstained. The lectin was found concentrated in the zones of junctions between hepatocytes (large arrows in A–D). The lectin was virtually absent from the areas where hepatocytes were not in contact (small arrows in C). H, hepatocytes; K, Kuppfer cell.

not reveal M_r markers (Fig. 4c, lane 7), and was completely abolished when incubations and washes were performed in the presence of 0.3M D-mannose, and not abolished in the presence of 0.3M D-galactose (not shown). Although it was difficult to obtain quantitative determinations, it was estimated (by densitometry) that the increase in staining was at least 40–60-fold higher than that found for the control cells. These results suggested that tumour cells displayed a complete change (overproduction) of CSL-binding glycans. Small differences were detected between the different tumour cells, but they were almost negligible when compared to the differences observed with normal cells.

In order to test whether this change was specific for CSL-binding glycans only, or was concerned with larger families of cell glycans, we performed Con A–HRP staining of the same samples. As shown in Fig. 4b, the staining profiles of tumour cells (Fig. 4b, lanes 2–6) differed qualitatively from that of control (Fig. 4b, lane 1). Significant qualitative differences were also observed between the various hepatoma cells. However, the global quantitative variations were much less pronounced between trans-

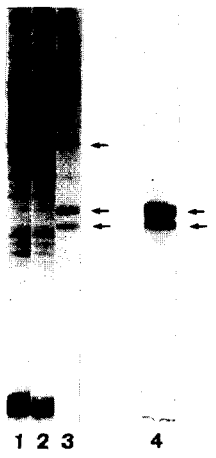

Fig. 3. Electrophoretic evidence (Coomassie Brilliant Blue staining, 15 μg of protein loaded in each lane) that CSL is related to an endogenous liver, D-mannose-binding lectin: (1) Total rat liver proteins, (2) rat liver proteins not bound to HRP-Sepharose columns, (3) proteins bound to HRP-Sepharose and specifically eluted in the presence of D-mannose, and (4) silver-protein staining of 5 μg of CSL, purified from rat cerebellum by immunoaffinity chromatography and used for detection of liver CSL ligands. In lane 3, note the presence of bands having identical M_r values as CSL (arrows).

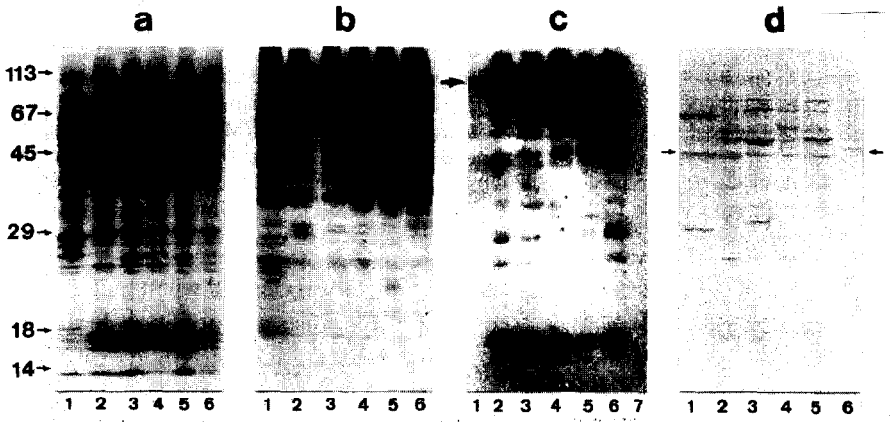

Fig. 4. Electrophoretic controls with : (a) Coomassie Brilliant Blue staining, (b) Con A–HRP staining, (c) iodinated-CSL staining, and (d) immunoblotting detection of CSL of normal and transformed hepatocytes. The following cells maintained for two days in culture have been analyzed: (1) Normal liver, (2) Yoshida ascite, (3) Zajdela ascite tumour, (4) Morris H35 hepatoma, (5) Zajdela hepatoma, (6) Reuter hepatoma. In (c), note the presence of only one positive band at 110 kDa (arrow) in the control and the huge increase in CSL-binding sites in tumour cells when compared to controls. In contrast, the differences in Con A–HRP staining (b) were much less pronounced. The arrows in (d) indicate the position of the 45-kDa band of CSL [bands with higher M_r values were also detected by omitting the anti-CSL antibody ($\times 10^3$) (not shown)]. Numbers on the left hand side of (a) indicate the M_r scale; 15 μg of protein was loaded in each lane.

formed and normal cells with Con A–HRP than with iodinated CSL. From the densitometric tracing, variation of the ratios of the levels of Con A–HRP staining per mg of protein in all samples analyzed was contained within a limit of 1.3.

When comparing the electrophoretic profiles revealed with Con A–HRP and ^{125}I-CSL, it appeared that the CSL-binding material was not proportional to that revealed by Con A–HRP. This particular observation could be explained easily by the greater selectivity of CSL for some special Con A-binding glycans.

Levels of CSL. — Special attention was drawn on the differential levels of CSL in the various cell types which had been analyzed by use of immunoblotting techniques. With this method and on this particular type of material only, the staining of bands having M_r values > 50 kDa was also detected by use of the second antibody only (Fig. 3d). This artefactual material was probably due to reacting IgG found in foetal calf serum used for the cultures. However, specific CSL-immunoreactive material was detected in all types of cells as a 45-kDa subunit of CSL. In controls (Fig. 4d, lane 1), the 31.5-kDa subunit of CSL was also detected. Sometimes, it was replaced by a band having a lower M_r value (Fig. 4d, lanes 2 and 4). This pattern found for cultured cells differed from the *in vivo* situation where three CSL subunits were detected (Fig. 3e). A specific shift of the expression of the 33-kDa subunit *in vitro* was possible but can also be explained by its rapid destruction by proteases in cultures. Nevertheless, it was evident that changes in levels of CSL were not as dramatic as for CSL ligands between controls and tumour cells.

DISCUSSION

The presence of lectin CSL in the cell bodies and in the junctions between hepatocytes constitutes a new finding. Whether CSL is actually hitherto an undiscovered liver lectin or not remains to be established. One of its subunits displays an M_r value identical to that of the calcium-dependent, D-mannose-binding protein (MBP) found in liver[43,44]. However, it is still obscure why the other CSL subunits have not been discovered.

The ubiquitous localization of CSL, both in the cytoplasm and in the areas of contact between certain cell types, is not a new feature. This has been previously shown in nervous tissue[14,16–18]. This duality of location seems to be a major fate of soluble lectins[45–47]. From its localization in tight junctions, it is tempting to postulate that CSL is playing a role in homotypic hepatocyte adhesions. The presence of one ligand of CSL in normal hepatocytes further sustains this contention.

In transformed cells, the level of CSL does not seem to be considerably increased or modified as shown by the immunoblotting technique. This is not the case for the ligands of CSL which are dramatically increased in transformed cells. The simultaneous increase in the number and amount of constituents binding to CSL suggested an overall change in the metabolism of CSL-binding glycans. This change, although dramatic, does not affect the metabolism of all glycans. This is evident with Con A-staining since the variations are relatively moderate. Thus, probably the modification affects the

synthesis of one particular type of minor glycans of the family of Con A-binding glycans, which are almost totally absent in the normal hepatocytes. This does not mean that they become major components in the transformed cells, but only that they are overproduced as compared to the normal cells.

The structure of these glycans is still unknown, but they are probably Con A-binding *N*-glycans. This assumption is based on the determination of the carbohydrate specificity of CSL[48] where the best ligands with a completely known structure belong to the series of oligomannoside glycans. But, as previously described, the best ligands of CSL present a more complex carbohydrate composition[48]. It is noteworthy that, in nervous tissue, some of the ligands of CSL are molecules implicated in cell adhesion, having epitopes recognized by anti-carbohydrate antibodies of the type HNK-1. For example, the myelin-associated glycoprotein (MAG), the major glycoprotein of the peripheral nervous system myelin (PO) having HNK-1 type glycans[49–52], and other low-M_r glycoproteins transiently expressed during cerebellar development binding to Elec-39 antibody (an antibody of the HNK-1 family[23,24]) are preferential ligands of CSL in nervous tissue[14,18]. There is evidence that the group of ligands recognized by CSL does not overlap exactly with the group of glycans binding to HNK-1 antibodies.

It is noteworthy that similar glycans (having the HNK-1 epitope) have been considered as markers of undifferentiated or transformed cells. The evidence presented in this report that an endogenous lectin is a receptor for ligands overexpressed in transformed cells does suggest that it may serve as a triggering event for cell transformation. In nervous tissue, we have shown that CSL is a cell-adhesion recognition lectin (CARL) which displays purely adhesive roles but also produces signals. This was evident for adhesion of oligodendrocytes where adhesion on CSL layers induces cell proliferation. In other situations, the signal is differentiation (neurite outgrowth for neuroblasts). Assuming that CSL plays a role in hepatocyte adhesion, with a resulting signal of differentiation (through only one ligand having M_r 100 kDa), the hepatocyte adhesion in transformed cells (through hundred different ligands) would result in a nonsense signal.

Whether this hypothesis is verified or not in other transformed cells remains an open question. Nevertheless, it suggests a new approach for understanding the role of glycans in cell transformation. The existence of a great diversity of endogenous lectins in various tissues[8–11] and changes in their pattern during malignant transformation[8,9,11] seem relevant of a more general role of the interactions between glycans and endogenous lectins in malignancy. This means isolation of endogenous lectins and of their respective ligands, and finally determining their structure with due comparison with those previously found in transformed cells[1,2,53–58].

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