Uptake and degradation *in vivo* and *in vitro* of laminin and nidogen by rat liver cells

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Laminin antigens are known to be present in the blood of normal individuals. In the present study we have investigated the fate of laminin-related molecules in the circulation. After intravenous injection in rats, the native laminin–nidogen complex, as well as the separated proteins, were rapidly eliminated from the blood (half-lives 2–10 min) by the liver. The large laminin fragments E1 and E8 (M_r 400 000 and 280 000 respectively), which contain the major cell-adhesion-promoting activities of laminin, were also cleared from the blood mainly by the liver, but the rate of uptake was an order of magnitude lower for these fragments than for laminin. This indicates that the uptake of laminin did not occur via cell-adhesion receptors. The endothelial cells of liver were the most important cell type in the uptake of laminin–nidogen complex, nidogen, laminin and fragment E1, whereas the parenchymal cells were responsible for more than 50 % of the uptake of E8 in the liver. Studies *in vitro* with cultured liver endothelial cells and parenchymal cells demonstrated that the ligands were endocytosed and degraded independently of plasma factors. The results reveal that the level of laminin antigens in blood is a very complex parameter. It is not only dependent on the turnover of basement membranes, but also on the degree of degradation of the material released into the blood and on the functional state of the liver, particularly the liver endothelial cells.

INTRODUCTION

Laminin and nidogen are the two quantitatively predominant non-collagenous proteins of basement membranes [for reviews, see Paulsson (1987) and Martin & Timpl (1987)]. In the tissue they are present as a 1:1 non-covalent complex, with nidogen bound to a short-arm structure of laminin (Paulsson *et al.*, 1987; Fig. 1). Such complexes are anchored into the basement membrane by additional non-covalent interactions (Yurchenco *et al.*, 1985; Charonis *et al.*, 1985; Laurie *et al.*, 1986; Paulsson, 1988). In addition to having structural functions, the laminin–nidogen complex also plays a role in mediating the attachment and spreading of cells on basement membranes. A number of cell-binding sites have been identified, and at present it appears that the most important ones are located on laminin and particularly on parts represented by the proteolytic fragments 1 and 8 (Timpl *et al.*, 1983; Aumailley *et al.*, 1987; Goodman *et al.*, 1987; Graf *et al.*, 1987), corresponding to the central portion of this cross-shaped molecule and the distal half of its long arm respectively (Fig. 1).

Basement membranes are subject to turnover, in particular when tissue is being remodelled during growth and wound healing. One route taken by released and partially degraded molecules is to the blood. Thus, with sensitive radioimmunoassays, it has been possible to measure laminin levels in serum and plasma. By using a radioimmunoassay for a particular laminin fragment, P2, a serum concentration of 83 ng/ml could be determined in normal rats (Risteli *et al.*, 1981). Much of the

Fig. 1. Schematic model of LN

Laminin fragments E1 and E8 are indicated by hatched areas.

laminin-related material detected with this assay was of large size and is likely to correspond to a heterogeneous set of structures, ranging from intact laminin to various proteinase-resistant laminin domains. As fragment P2 (M_r 45000; Rohde *et al.*, 1980) represents only a small structure within such larger laminin fragments, a true value for serum laminin could be 10-fold higher. Serum levels of laminin have more recently been investigated for their potential usefulness as markers for disease involving altered basement-membrane metabolism, such as diabetes (Risteli *et al.*, 1982), tumours (Brocks *et al.*, 1986), alcoholic liver disease (Niemelä *et al.*, 1985) and also other types of liver fibrosis (Gressner *et al.*, 1986). Even though changes in either synthesis or tissue degradation, followed by release to the blood stream, are likely to explain many of the observed variations in serum laminin, the clearance rate of circulating laminin and nidogen would also be expected to be an important factor. We have therefore studied the rates of elimination and degradation of intravenously injected laminin, nidogen and related structures, as well as the site of uptake at the organ and the cell level.

MATERIALS AND METHODS

Chemicals and animals

Laminin–nidogen complexes were purified from the mouse Engelbreth–Holm–Swarm tumour and dissociated into laminin and nidogen by treatment with 2 M-guanidinium chloride (Paulsson *et al.*, 1987). Laminin was also purified without the use of guanidinium chloride as described by Timpl *et al.* (1979). Fragment E1 was prepared by elastase digestion of laminin–nidogen complexes at 37 °C in the presence of Ca^{2+} (Paulsson *et al.*, 1988) and fragment E8 by mild elastase digestion at 25 °C in the presence of EDTA (Paulsson *et al.*, 1987; Paulsson, 1988). Fibronectin was purified from human plasma as described by Vuento & Vaheri (1979). Cellobiose, tyramine, cyanoborohydride, collagenase, cyanuric chloride, bovine serum albumin, and 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Na^{125}I was from The Radiochemical Centre, Amersham, Bucks., U.K. Culture medium RPMI 1640 supplemented with L-glutamine (2 mM), gentamycin (200 $\mu\text{g}/\text{ml}$) and Fungizone (50 $\mu\text{g}/\text{ml}$) was purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Male Sprague–Dawley rats, fed a standard diet and weighing about 200 g at the time they were killed, were obtained from Anticimex, Stockholm, Sweden.

Labelling of proteins

Incorporation of ^{125}I into tyrosine residues of proteins was carried out by the chloramine-T method, using Iodobeads from Pierce Chemical Co. (Rockford, IL, U.S.A.). The resulting radioactivity was approx. 10^6 c.p.m./ μg of protein. The synthesis of tyramine–cellobiose (TC) by reductive amination was as described by Smedsrød (1988a). ^{125}I -labelling of the TC adduct and coupling to proteins followed the method described by Pittman *et al.* (1983) with minor modifications (Smedsrød, 1988a). The integrity of the ligands after labelling with either method was ascertained by electrophoresis in SDS/polyacrylamide gels, followed by autoradiography (Johansson *et al.*, 1987). The amount of free ^{125}I radio-

activity in the labelled protein preparations was always below 10%.

Turnover and distribution *in vivo* of ^{125}I -labelled and ^{125}I -TC-labelled proteins

Rats were anaesthetized by intraperitoneal injection of phenobarbital (50 mg/kg), and ^{125}I -protein (10×10^6 c.p.m.) or ^{125}I -TC-protein (10^6 c.p.m.) was injected through the tail vein. Blood samples (25 μl) were collected from the tail tip in calibrated capillary tubes. Samples from rats injected with ^{125}I -protein were mixed with 0.5 ml of 20% trichloroacetic acid to precipitate macromolecular radioactivity, and radioactivity in the supernatant after centrifugation (trichloroacetic acid-soluble radioactivity) was taken as degraded protein. The amount of radioactivity in blood 1 min after injection was set to 100%. Organs from rats injected with ^{125}I -TC-protein were dissected out and analysed for radioactivity. Different types of liver cells were isolated as described below and their content of radioactivity determined. Cell numbers were assessed by visual counting of glutaraldehyde-fixed cultures in a phase-contrast microscope. Each '*in vivo*' experiment was performed on two or three different animals; results presented are from representative experiments.

Isolation and cultivation of liver cells

The method for preparation of pure cultures of functionally intact Kupffer cells, liver endothelial cells and parenchymal cells from a single rat liver was described previously (Smedsrød & Pertoft, 1985; Laakso & Smedsrød, 1987). Briefly, the liver was perfused with collagenase, and the resulting single-cell suspension was subjected to velocity and density centrifugations to produce pure suspensions of parenchymal cells and non-parenchymal cells respectively. Monolayer cultures of pure parenchymal cells were established and maintained in fibronectin-coated dishes. The suspension of non-parenchymal cells was a mixture of Kupffer cells, liver endothelial cells and stellate cells, essentially devoid of parenchymal cells, red blood cells and debris. Seeding of this cell suspension into dishes coated with glutaraldehyde-treated human albumin, followed by 15 min of incubation at 37 °C, resulted in attachment and spreading of Kupffer cells only. Unattached cells were transferred to fibronectin-coated dishes to enable attachment and spreading of liver endothelial cells. The average number of cells grown per cm^2 were 5×10^4 Kupffer cells, 2.5×10^5 liver endothelial cells, and 1×10^5 parenchymal cells. Contamination of Kupffer-cell cultures by liver endothelial cells and stellate cells was less than 1% and 10% respectively. Liver endothelial-cell cultures were contaminated by less than 1% and 10% Kupffer cells and stellate cells respectively. Cultures of either cell type were established and maintained in RPMI medium without serum. Identification of the different cell types was as described by Smedsrød *et al.* (1985).

Binding and endocytosis of labelled ligands by cultured liver cells

Cultures of liver endothelial cells and parenchymal cells were washed and supplied with fresh medium containing 1% bovine serum albumin and labelled proteins. Incubations with ^{125}I -labelled ligands were carried out for various lengths of time at 37 °C to measure endocytosis. Experiments were terminated by

transferring the media (600 μ l in 3.5 cm-diameter wells), along with 500 μ l of phosphate-buffered saline (PBS) used for washing of the cells to tubes containing 900 μ l of 20% (w/v) trichloroacetic acid. The amount of degraded ligand was quantified by measuring the radioactivities in pellet and supernatant obtained after centrifugation. Cell-associated radioactivity was determined after solubilization with 1% SDS/0.3 M-NaOH. Results presented are means of duplicate determinations, which did not differ by more than 10%.

RESULTS

Blood clearance and liver uptake of laminin, nidogen and laminin-nidogen complexes

The fate of laminin which enters the blood circulation was studied by injecting ^{125} I-labelled laminin in the tail vein of rats. Analysis of trichloroacetic acid-precipitable radioactivity in blood samples taken at various time points after injection showed that 50% of administered ^{125} I-laminin disappeared during the first 7 min (Fig. 2). The kinetics of the clearance was complex (Fig. 2b), indicating that more than one uptake mechanism was operating. After a lag period of 15–20 min, trichloroacetic acid-soluble products appeared in the blood (Fig. 2a),

Fig. 2. Uptake and degradation of intravenously injected ^{125} I-laminin

^{125} I-laminin (10^7 c.p.m.) was injected through the tail vein of a rat. Blood samples were collected into tubes containing trichloroacetic acid to precipitate non-degraded protein. Acid-precipitable radioactivity (intact ^{125} I-laminin; ■) measured after 1 min was taken as 100% (left ordinate of both a and b). Acid-soluble radioactivity (degraded ^{125} I-laminin; □) is given as a percentage of acid-soluble radioactivity measured at 1 min after injection (right ordinate of a). The time courses for clearance of intact laminin and the appearance of degradation products are shown in a linear fashion in (a). In (b) the clearance is shown in a semi-logarithmic plot to illustrate the complex elimination kinetics of laminin.

Fig. 3. Uptake and anatomical distribution of intravenously injected laminin (a), LN (b), and nidogen (c) labelled with ^{125} I-TC

^{125} I-TC-ligands (10^6 c.p.m.) were injected through the tail vein of rats. Blood samples were taken at various time points and processed as described in the legend of Fig. 2. The time course of clearance of intact ^{125} I-TC-ligands (■) and appearance of low- M_r ^{125} I-TC-labelled material (□) are shown (inset). Notice that the amount of low- M_r material does not increase with time, indicating that no degradation of ^{125} I-TC-labelled material is taking place. The distribution of ^{125} I-TC-ligands in various organs 30 min (a) and 60 min (b and c) after injection is shown in the bar diagrams. Results are presented as percentages of total radioactivity recovered (stippled bars) and as a percentage of the total radioactivity injected (open bars). See Fig. 2(a) for an explanation of the left and right ordinates of the insets.

suggesting ongoing endocytosis and intracellular degradation of the ligand followed by release of low- M_r products (i.e. amino acids) from the cells.

In order to determine the site of uptake, an ^{125} I-TC derivative of laminin was produced. Since this adduct is non-biodegradable, it will accumulate intralysosomally in cells which endocytose the carrier ligand (Pittman *et al.*, 1983). The kinetics of the elimination of ^{125} I-TC-laminin was closely similar to that observed for ^{125} I-laminin (Fig. 3a). However, ^{125} I-labelled trichloroacetic acid-soluble products in the blood were not detected, confirming that the degradation observed in Fig. 2 occurred intracellularly. At specified time points after administration of ^{125} I-TC-laminin the animal was killed

Fig. 4. Uptake and anatomical distribution of intravenously injected ^{125}I -TC-labelled E1 (a) and E8 (b)

The time course of uptake of intravenously injected ^{125}I -TC-E1 and E8, and their anatomical distribution after 2 h, were determined as described in the legend to Fig. 3. See Fig. 2(a) for an explanation of the left and right ordinates of the insets.

and the amount of radioactivity in various organs was determined. About 60% of the injected dose was recovered in the liver, and no other organ contained more than 7% (Fig. 3a). The overall recovery of injected radioactivity was about 90%.

Since laminin probably occurs as a complex with nidogen in basement membranes (Paulsson *et al.*, 1987), it was of interest to study how the association with nidogen affects the fate of laminin in the circulation. Intravenously injected ^{125}I -TC-derivatized laminin-nidogen complex (LN) was cleared from the blood at a slightly lower rate than laminin (Fig. 3b). The liver was the major site of uptake (Fig. 3b). However, in spite of the only minor difference in uptake observed between LN and laminin, nidogen was found to be recognized by the liver cells independently from laminin. Intravenously injected ^{125}I -TC-nidogen disappeared from the blood with a half-life of 2–3 min, and as much as 78% of injected radioactivity was recovered in the liver after 1 h (Fig. 3c). As shown for laminin (Figs. 2a and 3a), nidogen, radioiodinated by either of the two methods employed, was cleared from the blood at similar rates. Trichloroacetic acid-soluble radioactivity appeared in the circulation after 15–20 min only after injection of nidogen labelled by iodination of tyrosine residues (results not shown).

The finding that uncomplexed nidogen was taken up more efficiently than LN suggests that nidogen after dissociation exposes structures which are either shielded by laminin or generated by the potentially denaturing isolation procedure (2 M-guanidinium chloride). Since methods for isolation of nidogen under definitely non-denaturing conditions are not available at present, we could not discriminate between these two possibilities.

Fig. 5. Hepatocellular distribution of intravenously injected ^{125}I -TC-proteins

At 2 h after intravenous administration of the various ^{125}I -TC-proteins the cells of the liver were dispersed by collagenase perfusion, and liver endothelial cells (L), Kupffer cells (K), and parenchymal cells (P) were established in pure cultures. Content of ^{125}I is given either as c.p.m./ 10^6 cells, expressed in relative units, setting c.p.m. per 10^6 Kupffer cells equal to 1 (upper panel), or as c.p.m. per total cell population in the liver (lower panel), making use of the fact that the total numbers of Kupffer cells, liver endothelial cells and parenchymal cells in rat liver exist in the proportions 1:2.5:7.7 (Pertoft & Smedsrød, 1987). The proteins injected were laminin (Lam), LN, nidogen (Nd), E1 and E8.

However, uptake of laminin isolated by the same dissociation procedure was indistinguishable from that of native laminin (results not shown), indicating that exposure to guanidinium chloride had no effect on the uptake mechanism.

Blood clearance and liver uptake of laminin fragments with cell-binding activity

Since the laminin molecule is known to contain several different structural domains that are recognized by a variety of cells in cell-adhesion assays (Timpl *et al.*, 1983; Aumailley *et al.*, 1987; Goodman *et al.*, 1987; Graf *et al.*, 1987) the possible involvement of these cell-binding domains in the uptake of laminin from the circulation was investigated. Two major cell-binding fragments, denoted 'E1' and 'E8', were labelled with ^{125}I -TC and injected intravenously. Both these fragments have cell-attachment activity for liver parenchymal cells (S. Johansson, unpublished work). Compared with intact laminin, both fragments were removed very slowly from the blood. Except for the initial more rapid clearance of a fraction of each fragment, their half-lives in the circulation were more than 2 h (Fig. 4). After 2 h 42 and 49% of injected ^{125}I -TC-E1 and ^{125}I -TC-E8 respectively were present in the liver (Fig. 4).

Uptake *in vivo* in various liver-cell types

After intravenous injection, the distribution of the ^{125}I -TC labelled ligands was determined in isolated liver cells. Kupffer cells and liver endothelial cells were the

Table 1. Uptake and degradation *in vitro*

Laminin (Lam), nidogen (Nd) and laminin fragments E1 and E8 were radioiodinated on tyrosine residues and administered to monolayer cultures of liver endothelial and parenchymal cells. The amounts of degraded ('Degr.') and cell-associated ('Cell-assoc.') radioactivity were determined after 3 and 20 h of incubation as described in the Materials and methods section.

Ligand	Time (h)	Radioactivity (% of total)			
		Liver endothelial cells		Parenchymal cells	
		Cell-assoc.	Degr.	Cell-assoc.	Degr.
Lam	3	2.7	7.7	1.3	2.9
	20	3.1	18.1	2.9	3.9
Nd	3	6.4	27.6	3.1	1.9
	20	6.3	52.7	6.3	12.0
E1	3	1.2	2.9	0.4	1.2
	20	1.5	9.2	1.1	2.9
E8	3	1.4	2.5	1.1	5.2
	20	1.8	9.7	0.9	20.5

most active cells in the uptake of all the studied ligands (Fig. 5). Calculation of uptake in the total populations of the different liver-cell types revealed that the liver endothelial cells were the main site of uptake of LN, laminin, nidogen and E1, whereas the parenchymal cells were responsible for more than 50% of E8 accumulated in the liver (Fig. 5).

Uptake and degradation *in vitro* in various liver-cell types

To study whether plasma components were required for endocytosis, the ligands were incubated with pure cultures of parenchymal cells and liver endothelial cells under serum-free conditions. For this purpose the ligands were ^{125}I -labelled on tyrosine residues and the amount of trichloroacetic acid-soluble radioactivity in the culture medium was quantified. Table 1 shows that liver endothelial cells actively degrade the ligands with rates decreasing in the following order: nidogen > laminin > E1 = E8. Cultured parenchymal cells degraded E8 at a rate comparable with that of the endothelial cells, whereas laminin, nidogen and E1 were degraded at much lower rates by these cells. Essentially no trichloroacetic acid-soluble radioactivity was generated when a ^{125}I -labelled preparation of the very proteinase-sensitive fibronectin was incubated with the cells (results not shown), suggesting that degradation of the ligands was not due to proteolytic activity in the culture medium.

DISCUSSION

Laminin, nidogen and LN were all eliminated efficiently from the circulation, with half-lives ranging from 2 (nidogen) to 10 min (LN) (Figs. 2 and 3). The elimination kinetics were in each case complex, indicating the participation of more than one mechanism. The uptake was still almost exclusively in the liver (Fig. 3), with the liver endothelial cells and the Kupffer cells being about

equally active, but with the liver endothelial cells dominating the uptake because they are present in larger numbers (Fig. 5). These cells could also be shown to internalize and degrade laminin and nidogen actively (Table 1).

When the cell-binding fragments E1 and E8 of laminin were investigated a more complex situation became apparent. Each of these fragments did, after a rapid initial phase, show a very slow elimination (Fig. 4), the liver being the major organ of uptake. The rapid initial uptake may either reflect heterogeneities in the preparations of ligands or indicate saturating binding of the ligands to the receptors followed by a slow internalization. In the liver, fragment E1 was primarily taken up by liver endothelial cells and Kupffer cells, as was the case with intact laminin, whereas in the case of fragment E8 a significant proportion of the uptake was to parenchymal cells (Fig. 5). The parenchymal cells could also be shown to internalize and degrade fragment E8 actively (Table 1).

From this a number of conclusions can be drawn. Even though there are receptor mechanisms for uptake of both laminin and nidogen, these do not appear to act in a co-operative manner as the uptake of LN is not more rapid than the uptake of each component. On the other hand, the very slow elimination of fragment E1 and fragment E8 compared with laminin may indicate that receptor mechanisms for each part of the molecule act in a co-operative manner, favouring the uptake of the intact structure. Alternatively, the endocytosis of laminin by liver cells is mediated primarily by structures other than those present in the E1 and E8 domains.

Liver parenchymal cells have been shown to have attachment receptors for intact laminin and fragments E1 and E8 (Timpl *et al.*, 1983; S. Johansson, unpublished work). With the exception of fragment E8, these structures are not internalized and degraded to a significant extent by the parenchymal cells (Fig. 5). Furthermore, the cell-attachment-promoting activity of the isolated E1 and E8 fragments appears to be comparable with that of intact laminin for several cell types, including liver parenchymal cells (Aumailley *et al.*, 1987; Goodman *et al.*, 1987; S. Johansson, unpublished work). This would suggest that the receptor mechanisms involved in elimination of circulating laminin are distinct from the receptors operating in cell attachment. The nature of the clearance receptors is still unknown.

Our findings carry two major implications for the use of serum laminin as a disease marker. The first point is that, as the liver is the organ primarily responsible for clearance of circulating laminin, increased levels of serum laminin in alcoholic liver disease (Niemelä *et al.*, 1985) and liver fibrosis (Gressner *et al.*, 1986) could be due to a decreased clearance capacity because of impaired liver function rather than increased basement-membrane turnover. Secondly, the rate of elimination is highly dependent on the degree of integrity of the laminin, as already the rather large fragments E1 and E8 (M_r 400000 and 280000 respectively) are eliminated relatively slowly. Thus the serum levels of laminin-related antigen will not be dependent only on the amount of protein released to the circulation, but also on the extent of degradation of the material, which is likely to influence the rate of elimination. These aspects should be taken into account in the further evaluation of the clinical applicability of assays for serum laminin. It is also notable that the

central role of liver endothelial cells in the catabolism of several connective-tissue macromolecules (Smedsrød, 1988b) now has been extended to major basement-membrane components. The quantitative importance of this pathway relative to local catabolism of basement membrane proteins remains to be determined.

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