LIVER, BILIARY, AND PANCREAS

Galactosamine induced hepatitis induces a reduction in hepatocyte epidermal growth factor receptors

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Abstract

The rapid regenerative response of the rat liver to partial hepatectomy is associated with a decline in liver epidermal growth factor receptor numbers which implies that ligand epidermal growth factor receptor interactions maybe important in initiating and/or modulating this process. The proliferative process in toxic hepatitis (where in contrast with partial hepatectomy the majority of hepatocytes have been exposed to damaging influences) has been less widely investigated. We studied the DNA synthetic response of rat livers to toxic injury induced by a 350 or 800 mg/kg ip injection of galactosamine and that caused by 70% hepatectomy, comparing the changes in epidermal growth factor receptor status. Both resulted in down regulation of epidermal growth factor receptors, suggesting similar ligand epidermal growth factor receptor binding occurs during the proliferative response after galactosamine administration and after partial hepatectomy. In vitro studies on isolated hepatocytes showed that epidermal growth factor receptor down regulation was not a direct effect of galactosamine on hepatocyte membranes.

The liver has a remarkable capacity to regenerate after viral and toxic hepatitis, and surgical resection.¹ Hepatocyte proliferation takes place by division of existing adult hepatocytes, but the factors initiating this proliferation are complex and incompletely understood.² A significant early event in hepatic regeneration, studied in rats after 70% partial hepatectomy, is down regulation of the epidermal growth factor receptor.³⁻⁷ In vitro studies have clearly shown that the addition of the epidermal growth factor to hepatocyte membranes and hepatocytes causes epidermal growth factor receptor phosphorylation and down regulation, events which lead to stimulation of hepatocyte DNA synthesis implying that the epidermal growth factor receptor is involved in initiation of hepatic regeneration in vivo.⁵⁻⁸ TGF- α , a small polypeptide with a marked structural homology to epidermal growth factor also interacts with the epidermal growth factor receptor and can stimulate DNA synthesis in hepatocytes in culture.⁹⁻¹¹ Unlike the epidermal growth factor, generation of TGF- α has been shown within the

liver after partial hepatectomy, and therefore maybe the true mediator of the observed changes in the epidermal growth factor receptor.^{4,11,12}

Alternative models for investigating hepatic regeneration include chemical damage.^{13,14} D-galactosamine induces hepatitis within 24-48 hours of administration, producing histological damage similar to that of human viral hepatitis.¹⁵ The mode of action involves intracellular accumulation of galactosamine-1-phosphate and inhibition of uridine diphosphate-glucose pyrophosphatase which results in the depletion in uridine intermediates and subsequently depression of uracil nucleotide dependent synthesis of macromolecules – for example, mRNAs.¹⁶ It is not clear whether the regenerative processes after diffuse damage to the liver parenchyma are the same as those after partial resection of the liver. After resection the surviving cells are 'normal' whereas after toxic damage the surviving cells have been exposed to a chemical agent, and may show functional abnormalities. We therefore studied epidermal growth factor receptor down regulation after galactosamine induced hepatitis in rats, and compared those membrane changes with those induced by partial resection. In addition we studied the changes in epidermal growth factor receptors induced by galactosamine in primary cultures of rat hepatocytes and compared those with in vitro effects of the epidermal growth factor.

Methods**MATERIALS**

[6-³H]-thymidine (specific activity 25-30 Ci/mmol) and carrier free Na¹²⁵I were obtained from Amersham International PLC, Amersham, Bucks, UK. William's medium E, fetal bovine serum, penicillin/streptomycin, fungizone (amphotericin B), gentamycin, Nunclon microwell plates were obtained from Gibco Ltd, Paisley, Renfrewshire, Scotland, UK. Twenty four well Falcon tissue culture plates were from Flow Laboratories Ltd, Ayrshire, Scotland, UK. Insulin (human, monocomponent, Actrapid) was purchased from Novo, Copenhagen, Denmark. Collagenase (*Clostridium histolyticum*) was purchased from Boehringer Mannheim (UK) Ltd, East Sussex, UK. Other reagents and chemicals were from Sigma Chemical Company or BDH Chemicals, both of Poole, Dorset, UK. Murine epidermal growth factor (Collaborative

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Research, tissue culture grade) was obtained from Universal Biologicals, London, UK, or purified from mouse submaxillary glands according to the method of Savage and Cohen 1972.¹⁷

ANIMAL TREATMENT

All experiments were carried out on male August rats (250–300 g) supplied by the National Institute of Medical Research, Mill Hill, London, UK. They were maintained under conditions of controlled temperature and lighting with access to food and water *ad libitum*. All procedures were carried out under ether anaesthesia between 9 and 11 am. Seventy per cent hepatectomy was performed as described by Higgins and Anderson.¹⁸ Rats received a single ip injection of D-galactosamine, dissolved in saline, at 350 or 800 mg/kg body weight. Control rats received an equivalent injection of saline. Galactosamine treated rats were killed at either 24 or 48 hours and those after hepatectomy at 24 hours. One hour before being killed rats received an ip injection of ³H-thymidine (0.5 µCi/g body weight). Rats were killed one hour later by exsanguination under ether anaesthesia, the liver perfused *in situ* with ice cold saline and livers rapidly removed. Small sections of liver were formalin fixed for histology and frozen for measurement of thymidine incorporation, and the remaining tissue used to prepare liver cell plasma membranes. Blood samples taken were allowed to clot at 10°C for four hours, serum separated, and stored at –20°C for subsequent analysis of serum enzyme concentrations.

CELL CULTURE

Adult rat hepatocytes were isolated using a modification of the two step *in situ* collagenase perfusion of Berry and Friend.^{19,20} Eighty to ninety per cent of hepatocytes obtained were viable (trypan blue exclusion). Cells were resuspended at 3×10^5 or 2×10^5 viable cells/ml in William's E medium w/o L-glutamine, supplemented penicillin (200 IU/ml); streptomycin (200 IU/ml); gentamycin (80 µg/ml); fungizone (1.25 µg/ml); 5% (v/v) heat inactivated fetal bovine serum and dexamethazone (10^{-8} M) [plating medium] and seeded on rat tail collagen (type-1) coated plates, at densities of 75×10^3 /cm² in Falcon 24 well tissue culture plates or at 71×10^3 /cm² in Nucleon 96-well microwell plates. Cells were maintained at 37°C in an atmosphere of 5% CO₂/95% air with >95% humidity.

PLASMA MEMBRANE PREPARATION

Rat liver plasma membranes prepared by conventional methods Arnzonson and Touster (1974),²¹ Hubbard *et al* (1983).²² They were resuspended in 5 mM Hepes buffer pH 7.5 at approximately 5 mg/ml and stored in liquid nitrogen until used. Protein concentration was determined by a modification of the method of Lowry²³ with bovine serum albumin as a standard.

IODINATION OF EPIDERMAL GROWTH FACTOR

Epidermal growth factor was iodinated by a modification of the chloramine-T method, as previously described,²⁴ to give a specific activity of between 15 to 60 µCi/µg.

EPIDERMAL GROWTH FACTOR BINDING STUDIES

On hepatocytes

Binding studies on whole cells were carried out in 24 well tissue culture plates. After four hours allowed for the attachment of the cells the medium was removed and the new medium containing growth factors and/or galactosamine and/or uridine added at concentrations indicated in the Tables. At various times a plate of cells was cooled on ice, the medium removed, and cells washed two times with ice cold William's medium E containing 0.1% (w/v) bovine serum albumin and 20 mM Hepes (binding medium). Cells incubated with epidermal growth factor were washed twice with an acid buffer (50 mM glycine and 0.1 M sodium chloride, pH 4) between the binding medium washes to remove surface bound epidermal growth factor. Binding medium containing ¹²⁵I epidermal growth factor (approximately 4×10^5 cpm/0.5 ml) was then added to the cells which were incubated on ice for 2.5 hours. After this the cells were washed four times with ice cold Hanks balanced salt solution containing bovine serum albumin 0.1% (w/v) and treated twice with 0.6 ml of 0.2 M acetic acid containing NaCl (0.5 M) for six minutes. The acid washes were combined and the radioactive content determined in a gamma counter. Over 95% of surface bound radioactivity was removed from the cell in this way without removing iodine from epidermal growth factor. Internalisation of ¹²⁵I epidermal growth factor during the incubation at 4°C was minimal. Non-specific binding was determined at each point by inclusion of a 0.5 µg of unlabelled epidermal growth factor in replica wells. This was subtracted from total counts bound to obtain specific binding. Points represent the means of triplicate observations.

On plasma membranes

Increasing amounts of ¹²⁵I epidermal growth factor (50–3000 fmol) were incubated with between 60 and 100 µg membrane protein for 60 minutes at room temperature in Hepes buffer (20 mM pH 7.5) containing 0.1% (w/v) bovine serum albumin in a total volume of 0.2 ml in microfuge tubes. The tubes were then centrifuged for four minutes at 15 000 *g* in a microfuge and the supernatant and pellet separated. Both were counted in a gamma counter. Non-specific binding was determined in the presence of 1 µg unlabelled epidermal growth factor. Measurements were made in triplicate. Binding data were analysed by an iterative non-linear least squares correlation using the computer program GraphPAD Inplot and fitted to a 2 parameter model which identified a single low affinity binding site.

TABLE I DNA synthesis in the liver of rats in response to partial hepatectomy and galactosamine treatment

Treatment	³ H-thymidine incorporation (dpm × 10 ⁻³ /g liver)*	n
Sham/saline	9.1 (1.5)	6
Hepatectomy 24 h	535.7 (38.8)	6
GalN 24 h†	20.0 (1.4)	6
GalN 48 h†	229.0 (14.8)	6
GalN 48 h‡	80.7 (4.5)	4

*Mean (SEM); †GalN dose 800 mg/kg; ‡GalN dose 350 mg/kg.

ASSESSMENT OF DNA REPLICATION

In vitro

DNA synthesis was measured by assessment of ³H-thymidine incorporation into cell DNA. Twenty hours after seeding, the medium was changed and replaced with either plating medium alone, or plating medium containing insulin (10⁻⁷ M) and epidermal growth factor (20 ng/ml) with or without galactosamine (0.05–1 mM) and/or uridine (0.05–1 mM). ³H-thymidine (2 µCi/well) was included in the cultures from 28 hours after plating. The amount of ³H-thymidine incorporated was assessed biochemically as described by Selden and Hodgson (1989)¹⁹ between 44 and 48 hours after seeding. Each point was assayed in quadruplicate.

In vivo

³H-thymidine incorporation into liver DNA was measured biochemically by a modification of the method of Munro and Fleck 1966.²⁵ Liver (0.4 g) was homogenised in 5 ml ice cold 5 mM Hepes buffer pH 7.5 for 30 seconds with a Potter-Elvehjem homogeniser. The homogenate was mixed with 5 ml ice cold 0.6 M perchloric acid and allowed to stand for 10 minutes on ice. The precipitate was centrifuged at 1500 g for 20 minutes and the pellet washed twice with 0.2 M perchloric acid by repeated homogenisation and centrifugation. After the final wash the pellet was drained, solubilised in 4 ml 0.3 M KOH and incubated for 1.5 hours in a 37°C water bath. Samples were cooled on ice for 10 minutes and 4 ml 1.2 M perchloric acid added to reprecipitate. The tubes were left a further 10 minutes before centrifugation as above. The pellet was washed twice with 0.2 M perchloric acid and the final pellet containing DNA heated to 80°C for 20 minutes in 4 ml 0.5 M perchloric acid to hydrolyse DNA. Fifty microlitres of supernatant was counted for tritium in 1 ml Optiphase safe scintillant (Pharmacia-LKB).

Results

IN VIVO EFFECTS OF PARTIAL HEPATECTOMY AND GALACTOSAMINE LIVER PROLIFERATION
Preliminary experiments indicated that the maximum changes in epidermal growth factor binding and DNA synthesis occurred 24 hours after partial hepatectomy but 48 hours after galactosamine administration. Comparisons between the effect of these two procedures were made at the time of peak DNA synthesis.
Twenty four hours after hepatectomy there

Figure 1: Photomicrographs of liver sections 48 hours after galactosamine injections. (a) Autoradiography to show ³H-thymidine incorporation in hepatocyte nuclei (350 mg/kg). (b) A section showing wide spread areas of necrotic tissue after administration of 800 mg/kg galactosamine.

was a marked increase in DNA synthesis compared with sham operated animals. The mean increase in DNA synthesis, as assessed by ³H-thymidine incorporation into DNA, was approximately 60-fold (Table I).
In animals killed 48 hours after receiving 350 mg/kg body weight galactosamine there was a nine-fold increase in DNA synthesis compared with animals receiving saline alone (Table I).
In animals killed 48 hours after receiving 800 mg/kg galactosamine, the level of incorporation of ³H-thymidine into liver was 25-fold greater than in control animals. This increase had commenced by 24 hours, with levels of incorporation at this time being twice that in controls (Table I).
There was no difference in liver DNA ³H-thymidine incorporation at 48 hours between sham operated animals and those receiving saline.

LIVER DAMAGE
The histological appearance of tissue taken 48 hours after administration of galactosamine at a dose of 350 mg/kg showed the presence of diffusely scattered swollen and vacuolated hepatocytes. There was little lymphocytic cell infiltration. Incorporation of ³H-thymidine was predominantly into the nuclei of hepatocytes (Fig 1a). At a dose of 800 mg/kg body weight patchy necrotic areas were wide spread (Fig 1b).
Glutamic oxaloacetic transaminase (GOT/AST: EC2.6.1.1) concentrations were substan-

Figure 2: Effect of GalN treatment on liver plasma membrane EGF receptors. Binding of ¹²⁵I-EGF by plasma membranes isolated from the liver of rats 48 hours after administration of saline (○) or GalN, 350 mg/kg (●), 800 mg/kg (▲). Iterative non-linear least square correlation was used for curve fitting. Saturation binding curves and Scatchard analysis are shown and calculated parameters are in Table II. Membranes were prepared by the method of Aronson and Touster, 1974. Axis marked [EGF] pM represents measured free ligand concentration (pico molar) and that marked bound EGF fmol/mg protein. Regression analysis: r² values were above 0.98. Each point is the mean of triplicate measurements. The SE of each point was below 10%.

tially raised in galactosamine treated animals above rats receiving saline. At 48 hours after injection, serum concentrations of control animals were 81 (50–100) Sigma units/ml (mean and range for three animals), 182 (120–340) Sigma units/ml for animals receiving 350 mg/kg galactosamine, and 1570 (950–2160) Sigma units/ml for animals receiving 800 mg/kg galactosamine.

EPIDERMAL GROWTH FACTOR BINDING
Twenty four hours after partial hepatectomy, there was a 50% reduction in epidermal growth factor binding to liver plasma membranes with little change in epidermal growth factor receptor affinity, confirming previous observations.^{3,6} Epidermal growth factor binding to plasma membranes from livers of rats receiving 350 mg/kg galactosamine was reduced by 35–60% 48 hours after administration of the chemical, whereas the higher dose, of 800 mg/kg, reduced epidermal growth factor binding by >90% (Fig 2 and Table II). Galactosamine had no effect on the epidermal growth factor binding capacity of normal liver plasma membranes when included in the binding assay medium at 5 mM.

IN VITRO EFFECTS OF GALACTOSAMINE DNA SYNTHESIS
Exposure of hepatocytes to epidermal growth factor in combination with insulin resulted a

TABLE II Epidermal growth factor binding characteristics of plasma membranes from control and GalN-treated rats 48 hours after administration²²

Treatment	Bmax	Kd
Control	1656 (64)	493 (41)
GalN (350 mg/kg)	983 (70)	599 (86)
GalN (800 mg/kg)	776 (81)	1489 (237)

Bmax values are given in fmol/mg membrane protein (SE) and those of the Kd in pM (SE). Data were derived using the GraphPAD computer program for curve fitting.

TABLE III Effect of galactosamine and uridine on DNA synthesis of hepatocytes in culture

Treatment	³ H-thymidine incorporation (dpm × 10 ⁻³ /20 000 cells)
D	29.3 (2.4)
D I	43.2 (2.2)
D IE	79.2 (5.2)
D + G (1 mM)	15.7 (5.6)
D IE + G (1 mM)	25.9 (3.3)
D IE + G (0.5 mM)	48.4 (4.9)
D IE + G (0.05 mM)	77.5 (8.6)
D + U (0.5 mM)	28.5 (1.8)
D IE + U (5 mM)	35.0 (3.8)
D IE + U (0.5 mM)	92.9 (11.3)
D IE + U (0.05 mM)	93.0 (11.4)
D + G (1 mM) + U (0.5 mM)	33.6 (6.3)
D IE + G (1 mM) + U (5 mM)	53.8 (3.3)
D IE + G (1 mM) + U (0.5 mM)	76.9 (5.0)
D IE + G (1 mM) + U (0.05 mM)	66.5 (6.9)

Data are expressed as the mean and standard deviation (SD) of quadruplicated measurements from a typical experiment. The results were confirmed in two additional experiments. D = dexamethazone (10⁻⁸ M); E = EGF (20 ng/ml); I = insulin (10⁻⁷ M); G = galactosamine; U = uridine.

TABLE IV Effect of GalN by itself or in combination with uridine on ¹²⁵I-EGF binding to, and viability of, hepatocytes in culture

Treatment	¹²⁵ I-EGF bound (fmol/1.5 × 10 ⁵ cells) at different times after start of treatment [cell viability]		
	4 hours	20 hours	47 hours
Control	26.2 (0.4) [100]	14.1 (0.9) [>95]	10.2 (0.2) [>95]
GalN 1 mM	23.9 (0.6) [100]	12.3 (0.6) [>70]	4.4 (0.6) [<20]
GalN 1 mM + U 0.5 mM	22.5 (0.8) [100]	15.5 (0.8) [>95]	9.5 (0.4) [>90]
EGF (20 ng/ml)	6.05 (0.5) [100]	5.9 (0.3) [>95]	9.5 (0.7) [>95]

Cell viability was assessed using trypan blue. After removing the medium 0.5 ml of trypan blue (0.2% in Hank's balanced salt solution) was added to each well and left for two minutes. The trypan blue solution was then removed and cells that had taken up trypan blue estimated by microscopy using a graticule. Values are the mean (SD) of triplicate measurements.

marked enhancement of DNA synthesis assessed 26 hours later by ³H-thymidine incorporation. The addition of galactosamine (1 mM) to the cultures reduced the basal (unstimulated) levels DNA synthesis and completely blocked DNA synthesis induced by the combination of insulin and epidermal growth factor (stimulated). Galactosamine at 0.5 mM only partially reduced stimulated DNA synthesis. Uridine at a concentration which was not inhibitory to DNA synthesis (0.5 mM) restored basal and stimulated DNA synthesis to control levels (Table III).

VIABILITY AND EPIDERMAL GROWTH FACTOR BINDING

Galactosamine at a dose of 1 mM was directly toxic to primary hepatocyte cultures such that at 20 hours and 47 hours viability, as assessed by trypan blue exclusion was 70% and less than 20% respectively. In the presence of uridine (0.5 mM) cell viability remained at control levels. There was a 56% reduction in ¹²⁵I epidermal growth factor binding after 47 hours of culture in galactosamine (Table IV). Uridine prevented the fall in epidermal growth factor binding.

Discussion
In this experimental study we have shown that

liver plasma membranes isolated from animals in which galactosamine induced hepatic regeneration is occurring have a greatly reduced number of epidermal growth factor receptors. Sato *et al*²⁶ had addressed this question by indirect techniques, and shown by a perfusion system in intact liver that there was a reduced uptake of labelled epidermal growth factor by whole liver 48 hours after galactosamine treatment and also reduced epidermal growth factor binding by liver homogenates from galactosamine intoxicated rats. The study here clearly establishes the reduction in epidermal growth factor binding as a membrane event and avoids the inaccuracies caused by changes in volume of vascular compartments which may affect data derived from whole liver perfusion.

Broadly, two hypotheses could account for the reduction in epidermal growth factor binding by liver membranes from intoxicated rats. One possibility is that the galactosamine has directly or indirectly damaged the liver membranes, leading to loss of epidermal growth factor receptors. Alternatively, as occurs in the process of regeneration after partial hepatectomy, epidermal growth factor receptor internalisation may have occurred as an integral part of the process involved in initiating and modulating hepatocyte proliferation, a process probably reflecting production of TGF- α in the liver and binding of this ligand to the epidermal growth factor receptor.³⁴ Our *in vitro* studies have excluded a direct effect of galactosamine on liver membranes, as when metabolic damage was prevented by the co-addition of uridine to cultures, the epidermal growth factor binding capacity of hepatocytes was unaltered and their ability to proliferate in response to the combination of insulin and epidermal growth factor was unimpaired. The possibility that secondary changes in the hepatocyte membrane occur after the intracellular effects of galactosamine cannot be excluded, however, and unsurprisingly dead cells have diminished epidermal growth factor binding capacity. The histological appearance of the liver from which membranes were extracted 48 hours after galactosamine administration, while showing unequivocal hepatitis, did not show massive hepatic necrosis. Our data show that reduced epidermal growth factor binding to membranes occurs even with low doses of galactosamine, which have led to only scattered necrosis of hepatocytes and minor liver damage as exemplified by small rises in transaminase concentrations. Sato *et al*²⁶ comment that, in experiments in which doses of galactosamine twice those used in this study were administered, there was no significant reduction in the number of hepatic nuclei at 48 hours.

The experiments reported here therefore support the concept that regeneration after chemical damage to the liver involves similar mechanisms to those after partial hepatectomy – at least with regard to the modulation of the epidermal growth factor receptor. The timing of the major change in epidermal growth factor binding, later than that occurring after partial hepatectomy, is compatible with the concept that it is loss of hepatic mass after administration

of the chemical that is the stimulus to regeneration. It is of interest that similar changes in intrahepatic expression of the recently characterised hepatic growth factor occur in both toxic (CCl₄) and surgical induced damage of the rat liver, but again the changes in hepatic growth factor expression are earlier after hepatic resection than after chemical damage.²⁷ Such findings suggest that observations on the control of hepatocyte proliferation derived from the partial resection model may be extrapolated to the more complex problems of repair after chemical or viral hepatitis which are more relevant to disease in man.

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