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Rapid Communication

Epidermal growth factor protects against carbon tetrachloride-induced hepatic injury

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1. Epidermal growth factor (EGF) is known to protect the gastrointestinal tract against various noxious agents. Its potential value in preventing/treating hepatic injury is, however, largely unexplored. We therefore examined whether EGF could influence CCl_4-induced hepatic injury.

2. Female Sprague–Dawley rats (8 per group) received saline or recombinant EGF (500 or 750 μg/kg, intraperitoneal) 30 min before CCl_4 (20% v/v, in olive oil, intraperitoneal). Eighteen hours later, animals were killed, serum was collected for assay of biochemical markers of hepatic injury and livers were removed for histological analyses.

3. Administration of CCl_4 resulted in severe hepatic necrosis and caused a 10-fold rise in plasma alanine aminotransferase levels compared with levels seen in control animals (218 ± 15 compared with 23 ± 9 μmol/l in controls, mean ± SEM, $P < 0.01$). Serum malondialdehyde levels, used as a marker of lipid peroxidation, showed a 2-fold rise in response to CCl_4 treatment (median 4.0, quartile range 3.3–5.8 units/l compared with median 2.3, quartile range 2.1–2.5 units/l in controls, $P < 0.05$). Administration of EGF at 500 μg/kg, before the CCl_4, did not protect against injury, as assessed by histology or rise in plasma alanine aminotransferase levels. In contrast, animals given EGF at 750 μg/kg, before the CCl_4, had only minimal changes in histology, with only a minor rise in alanine aminotransferase levels (37 ± 4 compared with 23 ± 9 μmol/l in animals not given CCl_4) and had no significant rise in malondialdehyde levels.

4. EGF protects against CCl_4-induced hepatic injury and may provide a novel approach to the treatment of liver damage.

INTRODUCTION

Epidermal growth factor (EGF) is a 53 amino acid mitogenic peptide that is produced by the salivary glands and Brunners' glands of the duodenum. It is a potent stimulant of proliferation and healing of the gastrointestinal tract *in vitro* and *in vivo*, acting as cytoprotective agent, 'stabilizing' cells against noxious agents such as indomethacin [1]. There is therefore much interest in the potential clinical applications of recombinant EGF for the treatment of human gastrointestinal disease. EGF is also a potent stimulant of proliferation of hepatocytes *in vitro* (e.g.[1]), and may be involved in the regenerative process of the liver after partial hepatectomy, as removal of the salivary glands markedly delays re-growth [2]. Its potential value in the prevention/treatment of hepatic injury is, however, largely unexplored. Carbon tetrachloride (CCl_4) is a hepatotoxin which causes widespread damage to liver cells. Its administration is associated with increased lipid peroxidation, destruction of cytochrome *P*-450, fatty infiltration and hepatocyte necrosis [3]. In this study, we examine the potential beneficial effects of EGF in preventing hepatic injury induced by CCl_4.

MATERIALS AND METHODS

Human recombinant EGF

Commercially available, human recombinant EGF (Center for Genetic Engineering and Biotechnology, Havana, Cuba) produced in *Saccharomyces cerevisiae* was used for all experiments. The peptide was reconstituted in phosphate buffered solution, pH 7.4, and filter-sterilized (0.22 μm; Sartorius, Goettingen, Germany) before use.

Key words: Cytoprotection, hepatitis.

Abbreviations: ALAT, alanine aminotransferase; EGF, epidermal growth factor; i.p., intraperitoneal; MDA, malondialdehyde.

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Ethics

All animal experiments were performed under the appropriate regulatory guidelines of the Animal Welfare Committee of the Center for Genetic Engineering and Biotechnology, Havana, Cuba.

Animals

Female Sprague–Dawley rats (200–220 g) were obtained from the Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). Animals were individually located in wire-bottomed cages and acclimated for 7 days before the study and allowed food and water *ad libitum* throughout the experiment.

Experimental protocol

After an overnight fast, rats were randomized to groups of 8 and were given EGF [500 or 750 μg/kg, intraperitoneal (i.p.)] or saline, i.p. Thirty minutes later, all animals also received a single injection of CCl_4 (20% v/v, in olive oil; 1 ml/kg body wt, i.p.). An additional eight rats were also studied who did not receive EGF or CCl_4 (control group) but were treated in an identical fashion. Eighteen hours after CCl_4 administration, all animals were anaesthetized with diethyl ether, blood was collected by cardiac puncture (for subsequent serum biochemical marker assays) and the animals were then killed by cervical dislocation. Livers were harvested and fragments from the central lobe were flash-frozen and stored in liquid nitrogen before subsequent microscopic analyses.

Assay methods

Biochemical marker assays. Alanine aminotransferase activity (ALAT) was measured as an indicator of hepatocyte integrity and was determined using a commercially available test kit (Hoffmann-La Roche, Basel, Switzerland), based on the colorimetric test described by Reitman and Frankel [4]. Results are expressed as units/l.

Malondialdehyde (MDA) was used as a marker of lipid peroxidation and was evaluated using the thiobarbituric acid method [5]. Results are expressed as μmol/l.

Histological assessment

The liver fragments were sectioned at 5 μm and were stained with haematoxylin and eosin and Oil Red O to identify lipid droplets. Representative samples from each experimental group were placed onto the same slide to standardize staining conditions.

Statistical analyses

To establish homogeneity of variance for serum ALAT levels, data were $\log_{10}$ transformed before subsequent one-way analysis of variance. Where a significant effect was seen ($P<0.05$), individual comparisons were performed using *t*-testing based on the group means and residuals from the analysis of variance, a method equivalent to repeated measures analyses, which takes account of multiple comparisons being performed. Data are expressed as group mean ± SEM.

The data for MDA levels were not normally distributed and were therefore analysed using the non-parametric Kruskall–Wallis test. Where a significant difference was detected ($P<0.05$), multiple comparisons were performed using the non-parametric Dunn's test and Mann–Whitney U-test as appropriate. Data for MDA levels are expressed as median and interquartile range.

RESULTS

Rats treated with CCl_4 alone had severely damaged livers with extensive centrilobular zonal necrosis extending into the mid-zonal areas, with preferential location on the third Rappaport's space (Fig. 1, middle). Staining using Oil Red O showed a mild lipid infiltration characterized by small and scattered lipid droplets within the hepatocytes. These histological changes were associated with a 10-fold rise in serum ALAT activity levels compared with rats not given CCl_4 (Fig. 2, top; $P<0.01$) and a 2-fold rise in MDA activity (Fig. 2, bottom; $P<0.05$).

Administation of EGF at the lower dose (500 μg/kg) did not reduce CCl_4-induced hepatic injury, as assessed by histology. Enzyme levels were lower than those seen in animals given CCl_4 without EGF, although this difference did not reach statistical significance (Fig. 2).

In contrast, rats pre-treated with the higher dose of EGF (750 μg/kg) showed minimal changes in histology, with only occasional scattered individual necrotic hepatocytes (Fig. 1, bottom). Serum ALAT levels were significantly lower than those seen in animals given CCl_4 without EGF, and were only about 50% higher than levels seen in animals not given CCl_4 (Fig. 2, top). MDA levels were not significantly different to those seen in animals not given CCl_4 (Fig. 2, bottom).

DISCUSSION

Our studies examined the effect of EGF on the degree of hepatic injury induced by CCl_4, as assessed by histology and biochemical markers. EGF markedly reduced the degree of hepatic injury in a dose-dependent fashion.

The major sources of EGF production are in the

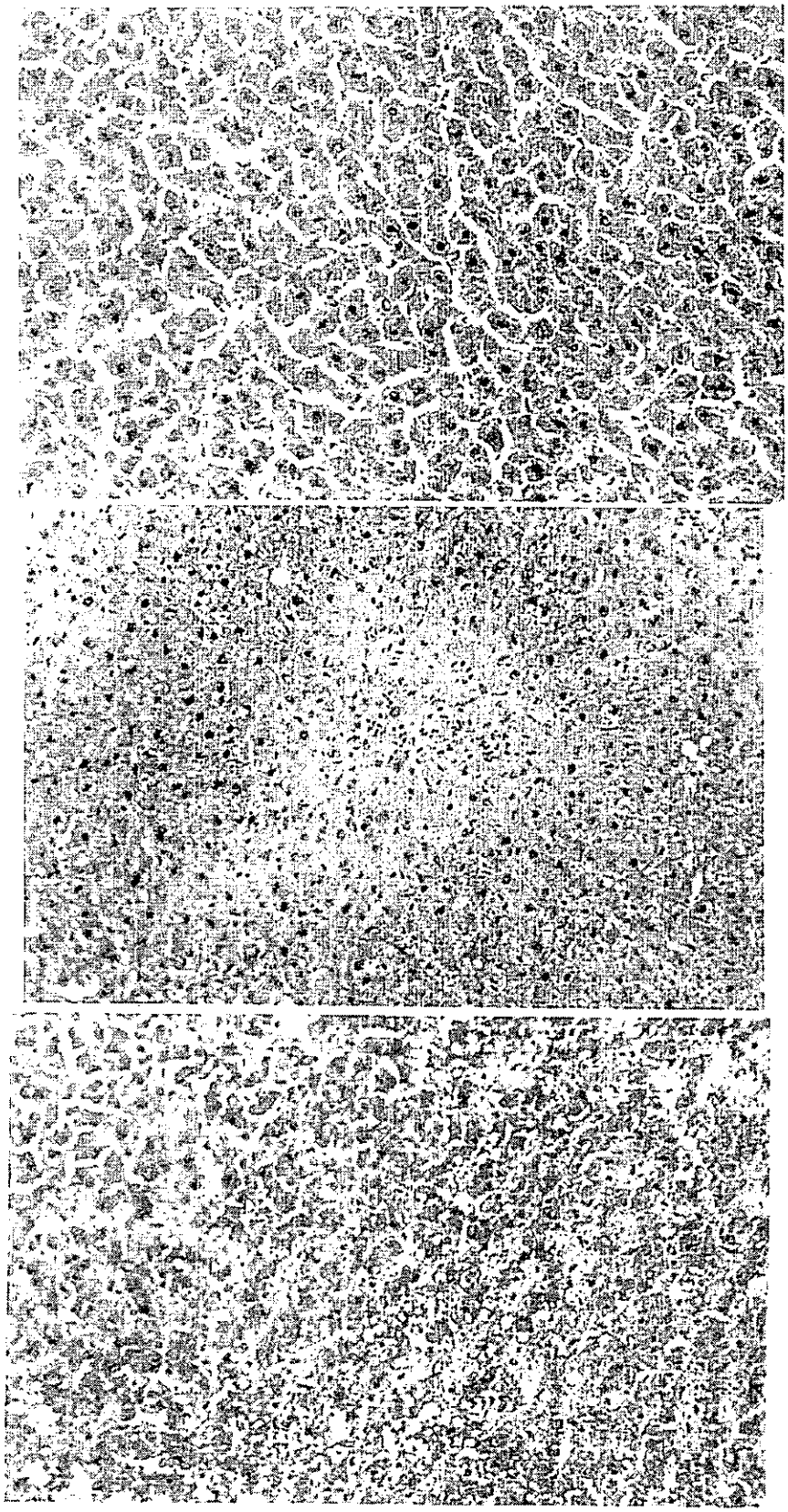

Fig. 1. Effect of EGF on CCl_4-induced liver injury as assessed by histology. Haematoxylin and eosin stain, magnification ×20. Top: control rats showing normal histology. Middle: rats treated with CCl_4 alone showed multiple large pale foci of necrotic hepatocytes: inflammatory cells are seen within the foci. Bottom: rats given EGF (750 mg/kg) before the CCl_4 had much less injury, showing isolated hepatocyte necrosis with swelling of surviving hepatocytes around the necrotic cells.

salivary glands, Brunner's glands of the duodenum and in the kidney. Although circulating levels of EGF are low, recent studies suggest that EGF might be important in stimulating regeneration of the injured liver; rats which have undergone partial hepatectomy have markedly delayed liver regeneration if their salivary glands have been removed [2]. Many studies have shown EGF to be a potent stimulant of growth for a variety of cell lines, including rat hepatocytes, and to act as a cytoprotective agent against gastrointestinal injury [1]. There is relatively little information, however, regarding its potential value for preventing hepatic injury.

Administration of CCl_4 is a well-established method of inducing hepatic necrosis [6]. Its hepatoxicity is probably related to free-radical production causing lipid peroxidation, as antioxidant agents can reduce CCl_4-induced injury [7]. Cleavage of a carbon–chloride bond from CCl_4 generates a trichloromethyl free radical $CCl_3^{\cdot}$, which in turn reacts with oxygen to form the highly reactive trichloromethyl peroxy radical ($CCl_3O_2^{\cdot}$). This radical can result in autocatalytic peroxidation of lipids within membranes [7, 8]. We therefore assayed serum MDA as a marker of this lipid peroxidation process and measured serum ALAT as a marker of hepatic necrosis.

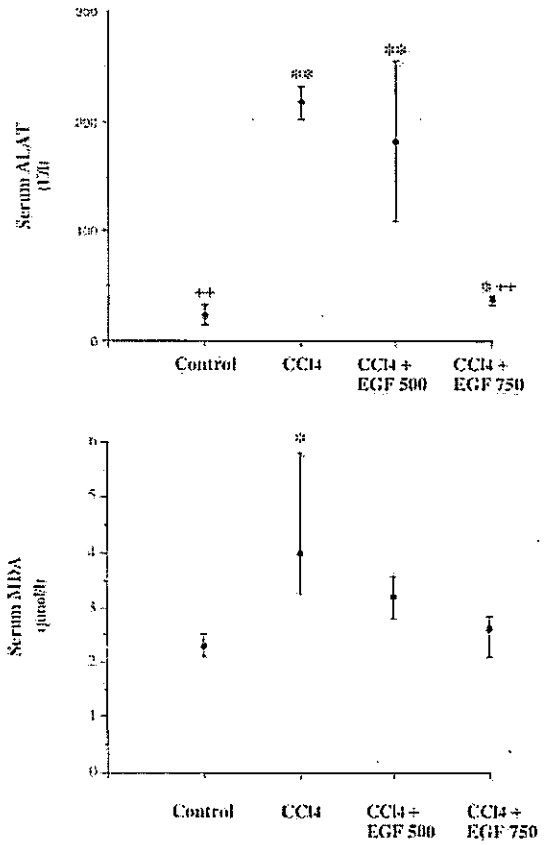

Fig. 2. Effect of EGF on CCl_4-induced liver injury as assessed by biochemical markers. Serum enzyme levels were assayed in various groups of rats ($n = 6$–8 per group). Groups were: control, animals given CCl_4 (20% v/v, in olive oil; 1 ml/kg, i.p.) and animals given CCl_4 plus EGF (500 or 750 μg/kg, i.p.). Top: serum ALAT levels, measured as a marker of hepatocyte injury, are expressed as mean ± SEM. Bottom: serum MDA levels, measured as a marker of lipid peroxidation, are expressed as median and interquartile range. * and ** signify $P<0.05$ and 0.01 compared with control animals. + and ++ signify $P<0.05$ and 0.01 compared with animals given CCl_4 alone.

The biochemical and histological data derived from these studies indicate that EGF is able to reduce markedly the injury induced by CCl_4 in a dose-dependent fashion. Our studies therefore support the previous finding that EGF can reduce hepatocyte injury after CCl_4 challenge *in vitro* [9]. The mechanism(s) by which EGF exerted this effect are unclear. Studies examining the effect of administering EGF to skin-burn sites [10], and to rat fetal lung cells stressed using hyperoxic toxicity [11], suggest that EGF stimulates the production of the antioxidant superoxide dismutase. EGF may therefore prevent CCl_4-induced hepatic injury by stimulating the production of superoxide dismutase in the hepatocytes. This idea is supported by the finding that administration of exogenous antioxidants can reduce CCl_4-induced hepatic damage [7]. Other mechanisms by which EGF might have exerted its effects include altering blood flow and prostaglandin levels, which have been shown to change in response to administration of EGF during studies on gastric models of injury.

EGF is found in human gastric juice at relatively high concentrations (about 500 ng/l). It probably acts as a 'luminal surveillance' peptide in the gastrointestinal tract, readily available to stimulate repair at sites of injury when the EGF receptors located on the basolateral membranes of the enterocytes become exposed [12]. In contrast, circulating concentrations of endogenous EGF are extremely low, suggesting that we are demonstrating a pharmacological, as opposed to a physiological, action of EGF on its receptor.

Clinical trials of EGF are presently underway for the treatment of ulcerative conditions of the bowel, for conditions such as necrotizing enterocolitis [13]. Doses used in this human trial (0.1 $\mu g \cdot h^{-1} \cdot kg^{-1}$ for 6 days, i.e. total dose 14.4 μg/kg) are much lower than those used in our rat study (a single injection of 750 μg/kg). The clinical safety of such high doses are therefore a potential cause for concern, particularly as administration of EGF to pigs at a dose of 30 $\mu g \cdot day^{-1} \cdot kg^{-1}$ for 28 days (total dose 840 μg/kg) resulted in ductal proliferation of the pancreas [14]. Caution must therefore be exercised if such high levels are to be used for any prolonged period in humans.

In conclusion, our studies suggest that EGF might provide a novel approach to the treatment of hepatic injury. Further research examining the potential benefit of EGF itself or other EGF receptor ligands, such as TGFα and amphiregulin, therefore seems warranted.

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REFERENCES

1. Playford RJ, Marchbank T, Calnan DP, et al. EGF is digested to smaller less active forms in acidic gastric juice. Gastroenterology 1995; 108: 92–101.
2. Jones DE, Tran-Patterson R, Cui DM, et al. Epidermal growth factor secreted from the salivary gland is necessary for liver regeneration. Am J Physiol 1995; 268: G872–8.
3. Srivastava SP, Singh KP, Saxena AK, Seth PK and Ray PK. *In vivo* protection by Protein A of hepatic microsomal mixed function oxidase system of CCl_4-administered rats. Biochem Pharmacol 1987; 36: 4055–8.
4. Reitman S, Frankel S. A colorimetric method for the determination of serum oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 1957; 28: 56–63.
5. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin Chim Acta 1978; 90: 37–43.
6. Bernacchi AS, Castro CR, Toranzo EG. Pyrazole prevention of CCl_4-induced ultrastructural changes in rat liver. Br J Exp Pathol 1980; 61: 505–10.
7. González R, Ancheta O, Pascual C, Pellón R, Frutos N, Milián V. Hepatoprotective properties of lobenzarit disodium in the rat. Biotechnol Apl 1991; 8: 140–7.
8. Pascual C, González R, Romay C. Drug effects on superoxide generation and chemiluminescence response of human leukocytes. Agents Actions 1991; 32: 277–82.
9. Wu B, Wan CW, Xu JR, Zhu JO. Effect of epidermal growth factor on cultured rat hepatocytes poisoned by CCl_4. Acta Pharmacol Sin 1997; 18: 176–9.
10. Kiyohara Y, Nishiguchi K, Komada F, et al. Cytoprotective effects of epidermal growth factor (EGF) ointment containing nafamostat, a protease inhibitor, on tissue damage at burn sites in rats. Biol Pharmaceut Bull 1993; 16: 1146–9.
11. Price LT, Chen Y, Frank L. Epidermal growth factor increases antioxidant enzyme and surfactant system development during hyperoxia and protects fetal lungs *in vitro* from hyperoxic toxicity. Pediatric Res 1993; 34: 577–85.
12. Playford RJ. Peptides and gastrointestinal mucosal integrity. Gut 1995; 37: 595–7.
13. Sullivan PB, Lewindon PJ, Oppenheimer SJ, Cheng C, Goodlad RG, Wright NA. A pilot study of the safety and efficacy of epidermal growth factor in the treatment of necrotising enterocolitis. Gut 1997; 41 (Suppl. 3): A69.
14. Vinter-Jensen L, Juhl CO, Teglbjaerg PS, Poulsen SS, Dajani EZ, Nexo E. Gastroenterology 1997; 113: 1367–74.