Epidermal growth factor reduces multiorgan failure induced by thioacetamide

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Abstract

Background—Multiorgan failure is a severe life threatening state where present therapeutic approaches are suboptimal. Epidermal growth factor (EGF) is a potent stimulant of repair in vitro and in vivo models. We therefore examined its potential beneficial effect in reducing mortality and injury induced by the noxious agent thioacetamide (TAA).

Methods—Mice (20 per group) were fasted overnight and received a single intraperitoneal dose of human recombinant EGF at 10 or 30 µg/kg or saline (control). Either 30 minutes before or after EGF, all animals also received TAA (40 mg/kg intraperitoneally). Twenty four hours later, surviving animals were killed, tissues collected, and degree of organ injury assessed.

Results—Fifty per cent (10/20) of control animals died within the first 24 hour period. Mortality was almost completely prevented by the higher dose of EGF whether given before or after TAA (p<0.01) and was reduced by about 50% with the lower dose of EGF. In control animals, the entire length of the jejunum and ileum had necrosis with or without mucosal denudation. In contrast, necrosis affected only about 10–20% of the total length in EGF treated groups (both p<0.01 vs control). Control animals showed marked glomerular tuft collapse, interstitial haemorrhage, and increased plasma creatinine levels. These effects were significantly reduced in animals given EGF (30 µg/kg; p<0.01). All groups showed similar changes in liver histology (centrilobular necrosis) and alanine transaminase levels (10-fold increase).

Conclusions—Although EGF did not prevent the hepatotoxicity associated with TAA, it reduced mortality, renal injury, and gastrointestinal damage. These studies provide preliminary evidence that EGF may be a novel approach for the prevention and/or treatment of multiorgan failure.

Keywords: gastrointestinal damage; nephrotoxicity; liver injury

Multiple organ failure (MOF) is a severe life threatening condition which usually occurs as a result of major trauma, burns, or fulminant infections. Whatever the initiating event, once established, MOF has a high mortality (up to 80%).1 The pathophysiological mechanisms underlying this condition are unclear although important contributory factors probably include hypoxia, increased intestinal permeability, bacterial translocation, endotoxaemia, and uncontrolled systemic inflammatory responses (see Nguyen and colleagues6).

Novel therapies that reduce the risk of patients developing MOF have direct clinical potential. Many of the patients who progress to MOF while an inpatient can be identified at an early stage, often at the time of admission, based on the severity of initial injury, for example, percentage of skin surface area affected in burns patients. There is therefore a potential therapeutic window where agents such as epidermal growth factor (EGF) could be administered prior to the development of the full MOF syndrome.

Thioacetamide (TAA) has been used extensively as an hepatotoxin to establish animal models of acute and chronic liver injury.1–3 Its injurious effects have been attributed, in part, to the biotransformation of TAA to the TAA sulphone and sulphoxide forms which react extensively with proteins, resulting in their denaturation.4–6 Although less well studied, administration of TAA can also result in damage to the kidney (particularly the proximal tubule7) and gastrointestinal tract,8 suggesting that administration of TAA might be a useful model of multiorgan failure.

EGF is a 53 amino acid peptide produced by the salivary glands and Brunner’s glands of the duodenum. It is a potent stimulant of proliferation and healing of the gastrointestinal tract, acting as a cytoprotective agent, “stabilising” cells against noxious agents such as indomethacin.9 In addition, we have recently shown that EGF can prevent hepatic injury induced by carbon tetrachloride.10 There is therefore interest in its potential clinical value for the treatment of human gastrointestinal and hepatic disease. We now report our studies examining whether EGF can influence the degree of renal, hepatic, and gastrointestinal injury caused by the noxious agent TAA.

Materials and methods

Animals

Adult male OF-1 male mice (20–22 g) were housed individually in wire bottomed cages and allowed free access to water and commer-

Abbreviations used in this paper: EGF; epidermal growth factor; hrEGF, human recombinant EGF; TAA; thioacetamide; MOF, multiple organ failure; ALAT, alanine aminotransferase.
EGF reduces thioaceticamide induced multiorgan failure

HGEGF was given to mice 30 minutes after
administration of TAA. The protocol used was as described
above except that EGF was given 30 minutes
after TAA instead of 30 minutes before. For
different sets of

For logistical reasons, two independent experi-
ments were performed on two separate occa-
sions. In each experiment, mice (10 per group) were
assigned randomly to receive saline or
eGF (10 or 30 µg/kg intraperitoneally) 30
minutes before receiving TAA (40 mg/kg intra-
peritoneally in a volume of 1 ml). After 24
hours, surviving animals were anaesthetised
lively with diethyl ether and blood collected via
he. The proximal 5 cm
of the small intestine (“duodenum”) was
lected for analysis and a further five (2 cm)
equi-spaced segments were then taken from the
remaining length of the small intestine, fixed in
buffered formalin, and subsequently processed for
morphological assessment (see below). The
boats. Animals were acclimatised for seven
days before experiments, which were per-
formed according to local regulatory
lines.

CHEMICALS
Human recombinant (hr) EGF, expressed in
Saccharomyces cerevisiae, was purchased from
HeberBiotc SA (Havana, Cuba). Prior to
administration, hrEGF was diluted in 0.9%
saline and sterilised via passage through a 0.22
µm filter. TAA was obtained from Sigma
Chemical Co. (St Louis, Missouri, USA) and
diluted in saline. All solutions were freshly pre-
pared before each experiment.

BIOCHEMICAL ASSAYS
Serum levels of alanine aminotransferase
(ALAT) were measured colorimetrically, according to the supplier's instructions (Hoffman
LaRoche, Basel, Switzerland). Serum levels of
creatinine were measured according to the
manufacturer's instructions (Reactivos SPIN-
REACT SA, Barcelona, Spain).

EXPERIMENTAL PROTOCOLS
Mice were fasted for 16 hours before commen-
cement of all experiments

Preliminary studies
A series of preliminary experiments were performed examining the effect on survival of various doses of TAA given alone. Groups of
10 mice were given 10, 20, 40, or 60 mg/kg of
TAA (single intraperitoneal injection) and were then observed for 48 hours. Mortality rates over this period were 10, 20, 60, and 100% for
animals given 10, 20, 40, and 60 mg/kg of TAA,
respectively, with virtually all deaths occurring 20–21 hours after administration of TAA. The dose of 40 mg/kg was chosen for subsequent studies as this was approximately the LD₅₀.

Effect of pretreatment with EGF on TAA toxicity
For logistical reasons, two independent experi-
m ents were performed on two separate occa-
sions. In each experiment, mice (10 per group) were
assigned randomly to receive saline or
eGF (10 or 30 µg/kg intraperitoneally) 30
minutes before receiving TAA (40 mg/kg intra-
peritoneally in a volume of 1 ml). After 24
hours, surviving animals were anaesthetised
lively with diethyl ether and blood collected via
cardiac puncture. Animals were then killed by
cervical dislocation and autopsies performed.

Stomachs were collected and inflated with 3 ml
of 10% buffered formalin. They were subse-
sequently opened along the greater curvature,
rinsed in saline, and examined for mucosal
damage using a dissecting microscope. The
small intestinal tract was dissected free, flushed
with 2 ml of normal saline, weighed, opened
along its length using dissecting forceps, and
macroscopically inspected. The proximal 5 cm
of the small intestine (“duodenum”) was
lected for analysis and a further five (2 cm)
equi-spaced segments were then taken from the
remaining length of the small intestine, fixed in
buffered formalin, and subsequently processed for morphological assessment (see below). The

kidneys, liver, large intestine, heart, lungs,
spleen, and pancreas were also collected and processed for histological assessment.

A further six mice, housed under identical circumstances, were not given TAA or EGF but were treated in an identical fashion to
determine locally validated “normal” values.

MICROSCOPIC ANALYSES
All histological and morphometric determina-
tions were performed in a blinded fashion.
Computer based image analysis was used to quantify small intestinal and hepatic damage.

Briefly, microscopic images were captured using a Sony DMC-107 camera, displayed on
an IBM computer via a VideoBlaster SE-100
frame grabber card. A 4x objective lens was
used for assessment of intestinal injury and a
25x lens for hepatic assessment.

For intestinal injury, each microscopic field
extended over 1500 µm and included approxi-
mately 25 villi and vertically sectioned crypts.
The entire length of the tissue samples was analysed using the DIGIPAT morphometric
image processing package (EICISOSF Calle
24 No. 408, Vedado, Havana, Cuba). The total
length of severely damaged mucosa, defined as
regions of frank necrosis, was then determined
and expressed as a percentage of the whole
length of tissue examined.

Hepatic tissues were treated in a similar
fashion with the images being captured and
quantitated using the same equipment and
software as that used for intestinal tissue. The
total area of injury in each fragment was deter-
mained in a standardised tissue area of 40 mm²,
by measuring and adding the size of the individual pericentral pale foci.

For renal tissue, the number of damaged
glomeruli and haemorrhagic foci per field were
determined using a 10x objective lens.

Effect of EGF on TAA toxicity when administered
after TAA
To examine the potential value of EGF when
given after TAA, two separate independent experi-
m ents were performed using identical
protocols with n=10 per group on each
occasion. The protocol used was as described
above except that EGF was given 30 minutes
after TAA instead of 30 minutes before. For
these studies, we also used a different set of
parameters to measure gut injury to show that
the apparent alteration in injury was not
dependent on using a particular assay method.

At the end of the study, the small intestine was
flushed with 2 ml of ice cold saline and the fluid
collected for subsequent haemoglobin assay
using a commercial kit (Reactivos SPINRE-
ACT SA, Barcelona, Spain) with the cyan-
omethaemoglobin method. The small intes-
tine was opened along its length, washed with
further cold saline, blotted with paper, and
weighed. It was then homogenised in phos-
phate buffered saline and aliquots taken to
determine total protein using the Lowry
method. Separate aliquots were used to deter-
mine total DNA content using a commercial
kit (GeneQuant, Pharcacia). Briefly, this was
achieved by digesting the tissue homogenate

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for four hours at 55°C in extraction buffer containing 1 M Tris pH 8, 0.5 M EDTA, 20% SDS, and 20 mg proteinase K. This was followed by three cycles of phenol/chloroform extraction and the DNA precipitated with ethanol. The pellet was dried and resuspended in 100 µl of TE for subsequent spectrophotometric quantitation at 260 nm. Results are expressed as the mean of three reading. Blood samples were collected by cardiac puncture for ALAT and creatinine assays.

STATISTICS

Data from the experiments using the same protocols on the two separate occasions were virtually identical, confirming the reproducibility of our findings. To demonstrate this reproducibility, we have shown the results for mortality data both as separate and cumulative results (tables 1, 2). Subsequent analyses of data for organ damage from the two occasions have been pooled for statistical comparisons. Mortality data were compared using a χ² test. As the data from biochemical and histological analyses were not normally distributed, data are presented as medians and interquartile ranges and statistical analyses used the non-parametric Mann-Whitney U test.

RESULTS

MORTALITY

In animals who died within the initial 24 hour observation period, deterioration in general condition was observed 16 hours after TAA administration with progressive lethargy, piloerection, and prostration. All deaths occurred 20–21 hours after TAA injection. For the study involving animals that received EGF prior to TAA, mortality was 50% in control animals, 20% in animals that had received the lower dose of EGF, and 0% in animals given the higher dose of EGF (table 1). EGF showed a similar protective effect in animals when administered after TAA (table 2). Postmortem examination showed large amounts of intraluminal bleeding in the small intestine with histological changes similar to those found in animals that survived. Morphometric analyses of small intestinal tissue were not performed in animals that died in this initial 24 hour period as postmortem changes may have influenced the results.

MACROSCOPIC OBSERVATIONS OF SURVIVING ANIMALS AT AUTOPSY

Control animals showed increased friability of the small intestine and liver. Many of the animals also showed evidence of intraluminal

Table 1 Twenty-four-hour survival values for rats given thioacetamide (TAA) (40 mg/kg) with or without epidermal growth factor (EGF) 30 minutes prior to administration of TAA. Ten mice were entered into each group on both experimental occasions.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TAA alone</th>
<th>TAA+EGF (10 µg/kg)</th>
<th>TAA+EGF (30 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/10 (40%)</td>
<td>8/10 (80%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>2</td>
<td>6/10 (60%)</td>
<td>8/10 (80%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Pooled data</td>
<td>10/20 (50%)</td>
<td>16/20 (80%)</td>
<td>20/20 (100%)</td>
</tr>
</tbody>
</table>

*p<0.05 v TAA alone.

Table 2 Twenty-four-hour survival values for rats given thioacetamide (TAA) with or without epidermal growth factor (EGF) 30 minutes after administration of TAA. Ten mice were entered into each group on both experimental occasions.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TAA alone</th>
<th>TAA+EGF (10 µg/kg)</th>
<th>TAA+EGF (30 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/10 (40%)</td>
<td>7/10 (70%)</td>
<td>9/10 (90%)</td>
</tr>
<tr>
<td>2</td>
<td>5/10 (50%)</td>
<td>8/10 (80%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Pooled data</td>
<td>9/20 (45%)</td>
<td>15/20 (75%)</td>
<td>19/20 (95%)*</td>
</tr>
</tbody>
</table>

*p<0.05 v TAA alone.

for four hours at 55°C in extraction buffer containing 1 M Tris pH 8, 0.5 M EDTA, 20% SDS, and 20 mg proteinase K. This was followed by three cycles of phenol/chloroform extraction and the DNA precipitated with ethanol. The pellet was dried and resuspended in 100 µl of TE for subsequent spectrophotometric quantitation at 260 nm. Results are expressed as the mean of three reading. Blood samples were collected by cardiac puncture for ALAT and creatinine assays.

STATISTICS

Data from the experiments using the same protocols on the two separate occasions were virtually identical, confirming the reproducibility of our findings. To demonstrate this reproducibility, we have shown the results for
bleeding into the small intestine. Similar changes were seen in the EGF treated animals although they were considered to be less severe qualitatively and this was confirmed quantitatively in animals given EGF after TAA (see below). Heart, lungs, spleen, pancreas, and kidneys appeared macroscopically normal.

FURTHER RESULTS FROM STUDIES WHERE EGF WAS GIVEN BEFORE TAA

Microscopic assessment

The major histological changes were present in the small intestine, liver, and kidney.

Intestine. Control animals had minimal histological changes in the duodenum. In contrast, the entire length of the jejunum and ileum showed extensive mucosal necrosis, usually associated with denudation (fig 1A). These changes were significantly reduced in both high and low dose EGF treated animals (fig 1B). Quantitative assessment showed that animals given TAA without EGF had mucosal necrosis of the jejunum and ileum affecting 100% of its length. In contrast, both doses of EGF significantly reduced the length of small intestinal tissue showing necrosis (both p<0.05 v TAA treated animals not given EGF; table 3). The large intestine was histologically normal in all groups.

Liver. Centrolobular necrosis, occasionally associated with haemorrhagic foci, were seen in all three groups (fig 2), often with some degree of inflammatory infiltration. Administration of TAA caused serum ALAT levels to increase by about 10-fold (p<0.01) (table 3). Pretreatment with EGF did not affect the amount of hepatic injury caused in response to administration of TAA, as assessed using histology or the increase in transaminase levels (table 3).

Kidneys. Control animals showed extensive collapse of the glomerular tufts (fig 3A). Minor degenerative acute changes were also found in the epithelial cells of the tubular system and haemorrhagic areas were also present in the medulla (fig 3B). These changes were reduced in both high and low dose EGF treated animals (fig 3C). Quantitation, as assessed by the number of collapsed glomerular tufts and haemorrhagic foci seen during microscopic examination, and changes in plasma creatinine levels, supported this finding of a protective effect of EGF. For all of these parameters, administration of either the high or low dose of EGF resulted in a significant reduction in the amount of injury by about 70–80% (table 3).

Discussion

We examined the effect of administration of high dose thioacetamide on multiple organ systems and determined the influence of EGF on the degree of injury sustained. Administration of TAA (40 mg/kg intraperitoneally) was associated with 50% mortality with the major sites of injury being the small intestine, liver, and to a lesser extent the kidneys. Administration of EGF either before or 30 minutes after TAA markedly reduced the mortality rate, intestinal injury, and renal damage in a dose dependent manner. However, administration of EGF had little effect on the degree of hepatic injury sustained, as assessed histologically and biochemically.

Administration of TAA is a well established method of inducing hepatic injury.3,4 Its toxic-
ity is probably related to biotransformation of TAA to the TAA sulphone and sulphoxide forms which react extensively with proteins, forming acetylimidolysine derivatives, resulting in their denaturation and charge modification. TAA may also injure cells by acting as a generator of free radicals, resulting in lipid peroxidation. Whatever the molecular mechanisms involved, administration of TAA results in increased apoptosis and necrosis, with necrosis being predominant when high doses of TAA are used. In the present series of studies, histological examination of intestinal tissue showed that necrotic changes were prominent throughout the small intestine. Necrosis was therefore likely to be the major final pathway of intestinal injury under these particular circumstances. Some degree of apoptosis does take place in this model, however, as shown by the presence of low levels of DNA laddering when collected intestinal residual DNA is run on agarose gels (CIGB, personal communication). Further examination of this process could potentially follow changes in apoptosis related enzymes such as caspasases but would be complicated by the fact that little viable mucosal tissue is left in a field containing high levels of necrotic tissue.

The hepatic changes seen in the present study (centrilobular necrosis and raised transaminase levels) have been described previously. It has also been noted that TAA can injure other organ systems, including the intestine and kidneys. Ortega et al found that chronic oral administration of TAA to rats in their drinking water caused enlargement of intercellular spaces of the jejunum and ileum with lymphocytic infiltration. Much more extensive changes were found in our studies using mice where virtually complete necrosis of the mucosa was seen. This difference in toxicity probably relates to the dosage used (40 mg/kg in the present study versus about 25 mg/kg) but may also relate to differences in the route of administration and species studied. In contrast with the study of Ortega et al, we examined the entire small intestine and found that the proximal gut (duodenum) was virtually unaffected by administration of TAA. The reason why the proximal bowel remained intact is unclear but may be due to enterohepatic circulation of toxic metabolites of TAA (which would result in relative sparing of the duodenum proximal to the ampulla), the requirement for pancreatic proteases to injure the intestine, or that the splanchnic blood flow to the duodenum was better preserved.

The predominant histological changes seen within the kidney were of tuft collapse with minor degenerative changes in the epithelial cells of the tubular system were seen. Original magnification x20. Bar=50 µm. (C) Pretreatment with EGF reduced the renal changes caused by TAA. Original magnification x20. Bar=50 µm.

Figure 3: Thiocacethamide (TAA) induced renal injury. Tissues were stained with haematoxylin and eosin. The renal tissue of TAA treated animals showed extensive collapse of the glomerular tufts. Original magnification x10. (B) Higher power photomicrograph of the same tissue demonstrating that only minor degenerative acute changes in the epithelial cells of the tubular system were seen. Original magnification x20. Bar=50 µm. (C) Pretreatment with EGF reduced the renal changes caused by TAA. Original magnification x20. Bar=50 µm.
interference with other key pathways involved in cellular respiration.

The major sources of EGF production are the salivary glands, Brunner’s glands of the duodenum, and the kidney. Many studies have shown EGF to be a potent stimulant of growth for various cell types in vitro and in vivo and that it acts as a cytoprotective agent against gastrointestinal injury caused by a variety of noxious agents such as non-steroidal anti-inflammatory drug induced gastric injury and trinitrobenzenesulphonic acid induced colitis. In addition, we have shown recently that exogenous EGF reduced carbon tetrachloride mediated hepatic damage, suggesting it might be of value in the prevention and treatment of hepatic injury. There is relatively little information, however, regarding its value for preventing/treating injury sustained in multiple organ systems, such as occurs during MOF.

The biochemical and histological data derived from these studies showed that EGF can markedly reduce intestinal and renal injury caused by TAA in a dose dependent fashion. The mechanism(s) by which EGF exerted these effects is, however, unclear. EGF influences multiple systems which might be important in mediating these effects, including upregulation of prostaglandin and mucus production, increasing mesenteric blood flow, and influencing stress associated protein kinases. Interestingly, EGF did not appear to influence the degree of hepatic injury yet markedly diminished mortality. It is therefore likely that the severe intestinal damage with intraluminal haemorrhage, caused by TAA, was the major factor that resulted in death of the animals.

Figure 4  Effect of epidermal growth factor (EGF) given 30 minutes after thioacetamide (TAA) on multiple organ systems. EGF reduced luminal bleeding (A), and the reduction in wet weight (B), DNA (C), and protein content (D) of the gut caused by TAA. The rise in plasma creatinine caused by TAA was also reduced by administration of EGF (E) although it did not influence the rise in serum alanine aminotransferase (ALAT) (F). *p<0.05, **p<0.01 compared with animals given TAA alone.
Clinical trials of EGF are presently underway for the treatment of ulcerative conditions of the bowel, such as necrotising enterocolitis.27 Our studies provide preliminary evidence that EGF may also be of benefit in MOF where increased gut permeability is thought to be an important component in its aetiology.2 MOF is a severe life threatening condition which usually occurs as a result of major trauma, burns, or fulminant infection.1 The pathophysiological mechanisms underlying MOF are unclear although important contributory factors probably include hypoxia, increased intestinal permeability, bacterial translocation, endotoxaemia, and uncontrolled systemic inflammatory responses.2 Whatever the initiating event, once established, MOF has a high mortality.1 Two of the most popular animal models used to induce MOF are administration of zymosan,28 which causes MOF by inducing pancreatic damage, and enterotoxin administration, which results in MOF via multiple mechanisms, including cardiovascular collapse.29 The present studies suggest TAA treatment, which results in MOF via multiple pancreatic damage, and enterotoxin administration, may be of benefit in MOF where EGF itself or other EGF receptor ligands, such as amphotericin B, induce injury provides a complimentary model which has its predominant initial toxic effects on the liver and small intestinal mucosa. Novel therapies that can reduce the risk of patients developing MOF have direct clinical potential. Many of the patients who progress to MOF while an inpatient can be identified at an early stage, often at the time of admission, based on the severity of initial injury, for example, percentage of skin surface area affected in burns patients.1,2 There is therefore a potential “therapeutic window” where agents such as EGF could be administered prior to the development of the full MOF syndrome. Further research examining the potential benefit of EGF itself or other EGF receptor ligands, such as transforming growth factor α, and amphotericin B, in the prevention of MOF and in the treatment of MOF once fully established, appear warranted.

We thank Dr K O’Reilly, Department of Histopathology, for histological advice. Funding was provided by the Medical Research Council, Wellcome Trust, and Royal Society.

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