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Role of Polymorphonuclear Leucocytes and Oxygen-derived Free Radicals in the Formation of Gastric Lesions Induced by HCl/ethanol, and a Possible Mechanism of Protection by Anti-ulcer Polysaccharide

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Abstract—This study examined the role of oxygen-derived free radicals in the pathogenesis of gastric mucosal lesions induced by HCl/ethanol. Superoxide dismutase, and catalase, and their combination reduced gastric lesion formation in mice. Gastric lesions were also reduced in mice treated with cyclophosphamide or anti-neutrophils, but not in mice treated with allopurinol or desulphated-carrageenan. Cobra venom factor did not reduce lesion formation. These results suggested that oxygen-free radicals may contribute to the formation of gastric mucosal lesions induced by HCl/ethanol, and that oxygen radicals were generated from neutrophils but not from xanthine oxidase. Anti-ulcer pectic polysaccharide, bupleuran 2IIc, which was recently isolated from the roots of *Bupleurum falcatum* L., showed potent inhibition of HCl/ethanol-induced gastric lesions in mice. Bupleuran 2IIc seemed to scavenge hydroxyl radical effectively. It was suggested that this anti-ulcer polysaccharide may provide protection to the gastric mucosa by scavenging oxygen-free radicals.

In our search for pharmacologically active compounds from crude drugs of plant origin, we recently found that the polysaccharide fraction (BR-2) of *Bupleurum falcatum* L. showed a potent inhibitory activity against HCl/ethanol-induced ulcerogenesis in mice and that the active polysaccharides, bupleurans 2IIb and 2IIc, were characterized (partial structures) as pectic polysaccharides (Yamada et al 1991a, b). BR-2 significantly protected against a wide variety of experimental gastric lesions, water immersion and restraint stress-induced, pylorus-ligated ulcer in mice or rats (Sun et al 1991).

The gastric mucosal protective action of this anti-ulcer polysaccharide cannot be explained by its protective coating effect alone, since it was effective not only by the oral route but also after intraperitoneal or subcutaneous administration, against HCl/ethanol-induced gastric mucosal lesion (Sun et al 1991). The exact mechanism of pathogenesis by HCl/ethanol is not yet known. Recent studies have demonstrated that active oxygen species might be involved in the formation of gastric mucosal lesions (Itoh & Guth 1985; Smith et al 1987; Yoshikawa et al 1990).

The present paper deals with the role of oxygen-derived free radicals in the pathogenesis of HCl/ethanol-induced lesions, the source of oxygen radicals, and the possible mechanism of the protection by anti-ulcer polysaccharide, bupleuran 2IIc.

Materials and Methods

Chemicals

Cu, Zn-superoxide dismutase (SOD) (from bovine erythrocytes, EC 1.15.1.1), catalase (from bovine liver, EC 1.11.1.6), xanthine oxidase (from buttermilk, EC 1.1.3.22), allopurinol, D-mannitol, xanthine, nitroblue tetrazolium (NBT), 2-deoxyribose, and ι-carrageenan were obtained from Sigma (St Louis, MO, USA). Cyclophosphamide was from Shionogi (Osaka, Japan). Lyophilized cobra venom factor (from *Naja haje*) was from Cordis Laboratories (Miami, FL, USA).

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Preparation of desulphated carrageenan

The desulphation of iota-carrageenan was prepared according to the methods of Ishizaka et al (1989). Briefly, 500 mg of ι-carrageenan was stirred with 0·06 M HCl in methanol (200 mL) at room temperature (21°C) for 24 h. Thereafter, the insoluble portion was separated down by centrifugation for 10 min at 3000 rev min^{-1} and dialysed against 0·9% NaCl (saline) for 24 h and then against distilled water for a further 48 h. The dialysates were lyophilized.

Preparation of anti-mouse neutrophil antibody

Anti-mouse polymorphonuclear leucocyte (neutrophils) antibody (anti-neutrophils) was obtained by immunizing rabbits according to the method of Ward & Cochrane (1965). New Zealand White rabbits were injected subcutaneously once weekly for 4 weeks with incomplete Freund's adjuvant containing mouse neutrophils (2×10^7 neutrophils/rabbit). The neutrophil preparation was obtained by instilling 2 mL thioglycollate medium intraperitoneally into mice, followed by peritoneal lavage with sterile saline 16 h later. The rabbits were exsanguinated 10 days after the last immunization, and the antisera obtained from the blood heat inactivated (56°C for 30 min) and then pooled. These anti-neutrophil sera were stored at −70°C until used.

Animals

Male ICR mice (SLC, Shizuoka, Japan) 8–10 weeks, 35–40 g, and male New Zealand White rabbits (SLC, Shizuoka,

Japan), 2·5 kg, were housed and maintained at 24 ± 1°C and constant humidity (55%).

HCl/ethanol-induced gastric mucosal membrane lesions

The method is based on the modifications of Mizui & Doteuchi (1983), and was described previously (Sun et al 1991; Yamada et al 1991a). The mice were divided into groups of 8–10. After 24 h fasting in wire-bottom cages, the mice received 0·2 mL 0·3 M HCl/60% ethanol (HCl/ethanol) orally. Each animal was killed by cervical vertebral dislocation 1 h after the administration of the HCl/ethanol, and the stomach was excised and inflated by injection of saline (2 mL). The ulcerated stomachs were fixed by 5% formalin for 30 min. After opening along the greater curvature, gastric damage visible to the naked eye was found in the gastric mucosa as elongated black-red lines (1–10 mm long × 0·5–1·5 mm wide) parallel to the long axis of the stomach in mice; this observation was similar to HCl/ethanol-induced lesions in rats. The lesion index was expressed as the sum of the length (mm) of all lesions in the fundic region.

Superoxide anion scavenging activity

The superoxide anion scavenging activity was determined according to the method of Beauchamp & Fridovich (1971) using the xanthine oxidase system. The reaction mixture consisted of 2·4 mL 50 mM sodium carbonate buffer (pH 9·2) and 0·1 mL each of xanthine (3 mM), BSA (0·15%), EDTA (4 mM), nitroblue tetrazolium (NBT, 0·75 mM), and test solution. The reaction was initiated by addition of 0·1 mL xanthine oxidase solution, and incubated at 37°C for 20 min. The reaction was terminated by adding 0·1 mL $CuCl_2$ (6 mM). The absorbance of formazan converted from NBT by superoxide anion was measured at 560 nm.

Hydroxyl radical scavenging activity

Hydroxyl radicals were produced by the interaction between chelated ferric iron and ascorbic acid according to the method of Cohen (1985), and hydroxyl radical scavenging activity was measured according to the methods of Grisham et al (1987). Briefly, hydroxyl radical formation was initiated by the addition of ascorbic acid (0·2 mM) to a 0·5 mL reacting fluid (pH 7·4) containing EDTA-Fe^{3+} (0·15 mM EDTA–0·10 mM $FeCl_3$), potassium phosphate (20 mM), and 2-deoxyribose (5 mM), and was incubated at 37°C for 20 min. Hydroxyl radical-mediated release of malondialdehyde from 2-deoxyribose was determined using the thiobarbituric acid method. The complex was extracted with 1·5 mL *n*-butanol/pyridine (15:1), and the absorbance of the organic layer was determined at 535 nm. Reduction in malondialdehyde formation in the presence of test sample was a measure of hydroxyl radical-scavenging activity.

Statistical analysis of data

Data obtained from pharmacological experiments are expressed as mean ± s.e.m. The differences between the control and the treatment in these experiments were tested for statistical significance by Student's *t*-test. A value of $P < 0·05$ was considered to indicate statistical significance.

Results

Effect of oxygen radical scavengers on the HCl/ethanol-induced gastric lesion

To assess the effects of superoxide dismutase (SOD), catalase, and combination of SOD and catalase on the acute gastric mucosal lesions induced by HCl/ethanol administration, SOD at a dose of 20 000 units g^{-1} or catalase at a dose of 40 000 units kg^{-1} was injected intravenously just before HCl/ethanol administration. D-Mannitol at a dose of 25 mg kg^{-1} (0·14 mmol kg^{-1}) was also injected intravenously before HCl/ethanol administration. Control animals received the same volume of vehicle (saline 0·2 mL). The vehicle did not influence the gastric mucosal lesions induced by HCl/ethanol (data not shown). The injury in the gastric mucosa induced by HCl/ethanol was significantly inhibited by treatment with superoxide dismutase or catalase (Table 1). Mannitol, a nonenzymatic oxygen radical scavenger, also significantly prevented this mucosal injury (Table 1). These results indicate that oxygen-derived free radicals may be involved in the pathogenesis of this mucosal damage.

Effect of allopurinol on the HCl/ethanol-induced gastric lesion

Allopurinol, an inhibitor of xanthine oxidase at a dose of 100 mg kg^{-1} (0·73 mmol kg^{-1}), was administered orally 48 and 24 h before the experiment in distilled water adjusted to pH 11 by the addition of 1 M NaOH. The control mice were given the same solvent (pH 11) alone. There was no difference in the gastric mucosal lesions between control and allopurinol-treated groups (Table 2). The results showed that allopurinol did not protect against gastric lesion formation induced by HCl/ethanol.

Effect of cyclophosphamide on the HCl/ethanol-induced gastric lesion

Cyclophosphamide, an immunosuppressive agent at a dose of 20 mg kg^{-1} (76·6 μmol kg^{-1}), was administered intraperitoneally 6, 4 and 2 days before the experiment. Control mice

Table 1. Effect of oxygen radical scavenger on HCl/ethanol-induced gastric mucosal lesions in mice.

Treatment	n	Lesion index (mm)	Inhibition (%)
Control (vehicle)	9	26·0 ± 3·1	—
SOD	9	9·4 ± 2·1	63·8***
Catalase	10	13·8 ± 2·9	46·9*
SOD + catalase	10	6·9 ± 1·2	73·5***
Mannitol	10	12·3 ± 2·7	52·5**

SOD (20 000 units kg^{-1}), catalase (40 000 units kg^{-1}), D-mannitol (25 mg kg^{-1}; 0·14 mmol kg^{-1}), and combined SOD (20 000 units kg^{-1}) and catalase (40 000 units kg^{-1}) or vehicle (saline) were injected intravenously. Expressed as mean ± s.e.m. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$.

Table 2. Effect of allopurinol on HCl/ethanol-induced gastric mucosal lesions in mice.

Treatment	n	Lesion index (mm)	Inhibition (%)
Control (vehicle)	20	19·3 ± 3·7	—
Allopurinol	21	24·2 ± 4·3	−25·5

Allopurinol (100 mg kg^{-1}; 0·73 mmol kg^{-1}) was administered orally 48 and 24 h before the experiment. Expressed as mean ± s.e.m.

Table 3. Effect of cyclophosphamide on HCl/ethanol-induced gastric mucosal lesions in mice.

Treatment	n	Lesion index (mm)	Inhibition (%)
Control	10	30·3 ± 6·8	—
Cyclophosphamide	11	8·4 ± 1·3	72·3**

Cyclophosphamide (20 mg kg^{-1}; 76·6 μmol kg^{-1}) was injected intraperitoneally 6, 4, and 2 days before the experiment. Expressed as mean ± s.e.m. $^{**}P < 0.01$.

received the same volume of saline (0·2 mL). The injury in the gastric mucosa induced by HCl/ethanol was significantly inhibited by pretreatment with cyclophosphamide (Table 3). Because it has been known that a decrease of circulating white blood cells is one major effect of cyclophosphamide, this result suggested that white blood cells (e.g. neutrophils, macrophages/monocytes), which retain high NADPH oxidase activity, may contribute to the formation of mucosal lesions.

Effect of desulphated carrageenan on the HCl/ethanol-induced gastric lesion

To induce depletion of macrophage/monocyte counts, desulphated carrageenan, which has potent specific toxicity against macrophages/monocytes, was used. Desulphated carrageenan, at a dose of 3 mg/mouse, was administered intraperitoneally 6, 4, and 2 days before the experiment. Control mice received the same volume of saline (0·2 mL). On desulphated carrageenan treatment, the number of circulating monocytes was decreased. Monocytes in white blood cells were 4·0 ± 1·2% in control and 1·0 ± 0·6% in desulphated-carrageenan-treated animals. However, there was no significant difference in the gastric mucosal lesions between control and desulphated carrageenan-treated animals (Table 4). This result indicated that macrophages/monocytes are not involved in mucosal injury induced by HCl/ethanol.

Role of neutrophils in HCl/ethanol-induced gastric lesion

In order to elucidate the role of neutrophils in the formation of injury, antibody was used to reduce the circulating neutrophils in mice. Anti-mouse neutrophil serum obtained from the immunized rabbits was injected intraperitoneally at a dose of 10 mL kg^{-1}, 19 h before the experiment. Normal rabbit serum was injected into control mice in the same manner. Pretreated mice with anti-neutrophil serum resulted in reduced neutrophil counts (neutropenia) (Table 5). This reduction of neutrophils prevented the formation of gastric mucosal injury (Table 6). The lesion indices in control and anti-mouse neutrophil serum-treated mice were 21·0 ± 2·5 and 8·8 ± 2·0, respectively. These results indicated that neutrophils are involved in mucosal injury induced by HCl/ethanol.

Table 4. Effect of desulphated carrageenan on HCl/ethanol-induced gastric mucosal lesions in mice.

Treatment	n	Lesion index (mm)	Inhibition (%)
Control	10	33·4 ± 5·6	—
Desulphated carrageenan	10	22·7 ± 4·9	32·0

Desulphaged carrageenan (3 mg/mouse) was injected intraperitoneally 6, 4, and 2 days before the experiment. Expressed as mean ± s.e.m.

Table 5. Effect of anti-neutrophil serum on circulating white blood cells (WBC) and neutrophils.

Treatment	n	WBC (cell mm^{-3})	Neutrophils (cell mm^{-3})
Control serum	10	6863 ± 893	1779 ± 285
Anti-serum	8	5006 ± 315*	780 ± 102**

Anti-mouse neutrophil serum was injected intraperitoneally at a dose of 10 mL kg^{-1} 19 h before the experiment. Expressed as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 6. Effect of reduced neutrophil counts induced with antibody on HCl/ethanol-induced gastric mucosal lesions in mice.

Treatment	n	Lesion index (mm)	Inhibition (%)
Control serum	12	21·0 ± 2·5	—
Anti-serum	11	8·8 ± 2·0	58·1**

Anti-mouse neturophil serum was injected intraperitoneally at a dose of 10 mg kg^{-1}, 19 h before the experiment. Expressed as mean ± s.e.m. $^{**}P < 0.01$.

Effect of depletion of complement component on the HCl/ethanol-induced gastric lesion

To assess the role of complement components in the ulceration, mice were depleted of complement components by injecting 5 units of anti-complementary cobra venom factor intravenously 24 h before ulceration. Although haemolytic activity was reduced to undetectable levels after treatment with cobra venom factor, both before and after ulceration (data not shown), this depletion did not alter gastric lesion incidence (Table 7). The lesion indices in control and complement-depleted mice were 29·4 ± 4·2 and 25·8 ± 4·1, respectively. This result indicated that complement activation is not involved in mucosal injury induced by HCl/ethanol.

Table 7. Effect of cobra venom factor on HCl/ethanol-induced gastric mucosal lesions in mice.

Treatment	n	Lesion index (mm)	Inhibition (%)
Control	10	29·4 ± 4·2	—
Cobra venom factor	10	25·8 ± 4·1	12·2

The mice were given 5 units of cobra venom factor intravenously 24 h before the experiment. Expressed as mean ± s.e.m.

Superoxide anion scavenging activity of polysaccharide

The anti-ulcer polysaccharide, bupleuran 2IIc, did not inhibit the reduction of NBT which was mediated by superoxide anion in a concentration of up to 10 mg mL^{-1} (data not shown). This result indicates that the anti-ulcer polysaccharide is neither a scavenger of superoxide anion nor an inhibitor of xanthine oxidase.

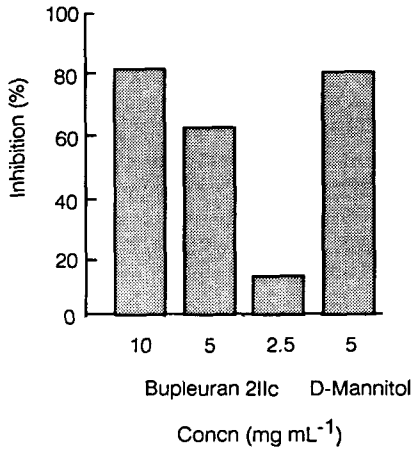

FIG. 1. Effect of anti-ulcer polysaccharide on hydroxyl radical-mediated production of malondialdehyde from 2-deoxyribose. The data reflect mean of two separate experiments.

Hydroxyl radical scavenging activity

Bupleuran 2IIc effectively scavenged hydroxyl radical which was measured by the release of malondialdehyde from 2-deoxyribose (Fig. 1). The effective concentration required for a 50% reduction in malondialdehyde production was 5 mg mL^{-1}.

Discussion

HCl/ethanol-induced gastric mucosal lesion is often used for screening cytoprotective and anti-ulcer agents. It has been suggested that oxygen radicals may contribute to the formation of HCl/ethanol-induced gastric lesions, since antioxidants such as polyamines and non-protein sulphydryls have protective potency against gastric damage induced by HCl/ethanol (Mizui & Doteuchi 1983; Mizui et al 1987a).

The present results have demonstrated that intravenous administration of SOD, catalase, and a combination of SOD and catalase significantly and remarkably reduced the gastric lesion induced by HCl/ethanol. Since SOD and catalase are highly specific in their respective catalytic mode of action, these results indicate that oxygen-derived free radicals may be involved in the pathogenesis of mucosal damage.

Despite the enzyme specificities of SOD and catalase, it is not possible to determine unambiguously from pharmacological data alone which is the important radical species involved (Halliwell & Gutteridge 1985). However, protection of mucosal damage in this model by the action of D-mannitol suggested that the hydroxyl radical produced by interconversion of superoxide anion might be involved in this pathogenesis. Although dimethylsulphoxide, a hydroxyl radical scavenger, has been used to determine whether hydroxyl radicals are involved in the pathogenesis of mucosal damage (Itoh & Guth 1985; Perry et al 1986; Mizui et al 1987b; Szelenyi & Brune 1988; Terano et al 1989), we used D-mannitol for the pretreatment as dimethylsulphoxide has protective properties (anti-inflammatory, analgesia and vasodilation), and has been shown to inhibit neutrophil chemotaxis, adherence, superoxide production, and platelet aggregation (Antony et al 1983; Jacob & Herschler 1986; Beilke et al 1987; Sekizuka et al 1989; Tarnasky et al 1990).

Pretreatment with allopurinol, a competitive inhibitor of xanthine oxidase, has previously been shown to be protective against the lesions induced by haemorrhagic shock, burn shock, treatment with compound 48/80, ischaemia-reperfusion or ethanol (Terano et al 1989; Yoshikawa et al 1990). The present study has shown that mice pretreated with allopurinol were not significantly different from control animals, indicating that xanthine oxidase is not involved in this lesion formation. In contrast, reduction of neutrophils with anti-neutrophil serum, but not depletion of monocyte with desulphated carrageenan, reduced gastric injury significantly. These results suggest that oxygen radicals generated from neutrophils play important roles in gastric mucosal lesions in mice induced by HCl/ethanol. Although the mechanism by which HCl/ethanol activates neutrophils is unclear, it has been reported that xanthine oxidase-derived oxy-radicals are implicated in neutrophil-mediated injury to gastrointestinal mucosa (Grisham et al 1986). Xanthine oxidase-derived oxy-radicals appear to activate and attract neutrophils into the mucosa, and the activated neutrophils subsequently injure the microvasculature via the release of oxy-radicals or proteases (Hernandez et al 1987). However, the present results did not support the hypothesis that xanthine oxidase-derived oxy-radicals are involved in HCl/ethanol-induced injury since the xanthine oxidase inhibitor, allopurinol, did not prevent HCl/ethanol-induced injury (Table 2). Although complement activation products, C5a, can stimulate neutrophils, and the resulting cells generate oxy-radicals, the present results using cobra venom factor did not support involvement of the complement system in HCl/ethanol-induced injury.

Previously, we reported that the anti-ulcer polysaccharides isolated from *B. falcatum* L. have potent protective and anti-secretory activities, and that the mechanism of gastric mucosal protection by orally administered polysaccharide may be due to reinforcement of resistance of the mucosal barrier by a protective coating (Sun et al 1991). Present results demonstrate that an anti-ulcer polysaccharide, bupleuran 2IIc, is an effective scavenger of hydroxyl radical generated by the interaction between Fe^{3+} and ascorbic acid (Fenton reaction). Therefore, in addition to the protective coating effect, the oxygen radical-scavenging activity seems to be the most likely mode of action of the anti-ulcer polysaccharides. Certain carbohydrates and related compounds (e.g. glucose and mannitol) are known to scavenge hydroxyl radicals. Bupleuran 2IIc is a carbohydrate polymer (Yamada et al 1991a), and may act as a scavenger. It has been reported that pectin reacts with hydroxyl radicals (Gilbert et al 1984; Uchida & Kawakishi 1986), and a possible scavenging mechanism has been clarified by electron spin resonance (Gilbert et al 1984); bupleuran 2IIc is a pectin-like polysaccharide (Yamada et al 1991b), and it may be hypothesized that the same reaction mechanism might contribute to the scavenging activity of bupleuran 2IIc.

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