Corticosteroids reduce regenerative repair of epithelium in experimental gastric ulcers

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Abstract

The association between corticosteroid treatment and gastric ulcer healing is controversial. The effects of corticosteroids on experimental ulcer healing in the rat were studied and the effect of coadministration of a prostaglandin E_1 analogue was determined. Forty male adult rats were divided into five groups and pretreated for 10 days as follows: (a) control, (b) prednisolone (10 mg/kg), (c) prednisolone and misoprostol (300 µg/kg), (d) misoprostol, and (e) indomethacin (2 mg/kg) Gastric ulcer was induced by applying a cryoprobe to the serosal surface of the stomach. Healing was assessed by determining the ulcer size at three and six days. Mucosal regenerative activity at the ulcer edge was assessed by quantitating DNA synthesis in cells by immunohistochemical techniques using monoclonal antibodies to detect expression of proliferating cell nuclear antigen (PCNA) and incorporated 5-bromo-5-iododeoxyuridine (BrdU). Compared with control rats, the rate of healing and the mucosal regenerative activity were significantly reduced in rats treated with prednisolone and indomethacin ($p<0·05$). Coadministration of misoprostol and corticosteroids reversed the delay in healing and the reduction in mucosal regeneration induced by corticosteroids alone. With misoprostol alone, the ulcer size and the number of epithelial cells that actively synthesised DNA did not differ from control animals. A comparison between the two immunohistochemical markers of cell proliferation showed a highly significant correlation between the two techniques ($r=0·64$, $p<0·005$), indicating that the simpler PCNA technique should prove valuable in assessing regeneration in experimental ulcer disease.

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The interaction between adrenocorticosteroid treatment and clinical manifestations of ulcer disease in man is controversial. In a classic analysis of prospective studies of adrenocorticosteroid and adrenocorticotrophic hormone therapy, Conn and Blitzer[1] concluded that overall the frequency of peptic ulcer was not noticeably increased during therapy, although upper gastrointestinal haemorrhage was more common in patients taking corticosteroids. In addition, peptic ulcer appeared to be more common in patients who had received a large total dose of prednisolone (>1000 mg) and those who had been treated for more than 30 days. More recently, Messer *et al*[2] re-examined the association between corticosteroid therapy and peptic ulceration or gastrointestinal haemorrhage in 71 controlled clinical trials. They concluded that there was an increased risk of peptic ulceration (relative risk 2·3, 95% confident interval 1·4, 3·7) and haemorrhage in patients taking corticosteroids.

These observations result in hypotheses about the mechanism of the increased incidence and complication rates of peptic ulcers and also call for strategies to prevent ulcer development and enhance healing. Our recent studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs), another class of drugs commonly associated with gastroduodenal complications, depress the processes of epithelial cell proliferation that leads to ulcer healing. The coadministration of a prostaglandin E_1 analogue (PGE_1), however, returned to normal the rates of experimental ulcer healing and of cell proliferation in the proliferative compartment of the gastric crypts adjacent to the ulcer edge.[3]

We examined the theory that the healing of ulcers would be slowed by corticosteroid therapy, and investigated the effect of this treatment on both ulcer size and the rate of regenerative repair at the ulcer edge in gastric ulcers induced by cryoprobe in the rat stomach. In addition, we studied the effect of the PGE_1 analogue misoprostol on ulcer healing in rats given corticosteroids. For comparison we included a group of animals treated with indomethacin, a known inhibitor of regenerative repair at the edges of cryo-ulcers.

Studies of cell proliferation during ulcer healing have generally measured DNA synthesis by methods such as [3H]-thymidine labelling followed by autoradiography, or immunohistochemistry for the incorporated uridine analogue 5-bromo-2-iododeoxyuridine (BrdU). Although these methods are accurate, they are time consuming and cumbersome. Proliferating cell nuclear antigen (PCNA) is a nuclear protein that is expressed maximally at the time of the 'S' phase and can be used to identify proliferating cells by immunostaining. It has the advantage of being detectable in formalin fixed paraffin sections. We also compared the use of the two immunohistochemical techniques, using antibodies for PCNA and for incorporated BrdU, for quantitative detection of mucosal cell proliferation.

Methods

Forty adult Wistar rats weighing 200–250 g were used. Animals were given free access to food and water during the treatment period. The PGE_1 analogue, misoprostol was supplied by G D Searle and Co Ltd.

TREATMENT GROUPS

Animals were divided into five groups of eight. They were treated daily at 9.30 am starting 10 days before ulcer induction and continuing until they were killed. The groups were treated as follows:

- Group A: phosphate buffer (pH 7·4) by gavage, and sodium bicarbonate (5%) by subcutaneous injection (controls);
- Group B: indomethacin (2 mg/kg) by subcutaneous injection and gavage vehicle;
- Group C: prednisolone (10 mg/kg) by subcutaneous injection and gavage vehicle;
- Group D: prednisolone (10 mg/kg) by subcutaneous injection and misoprostol (300 μg/kg) by gavage;
- Group E: misoprostol (300 μg/kg) by gavage and sodium carbonate by subcutaneous injection.

The dosages of indomethacin and the PGE_1 analogue were those given in the study of Levi *et al.*[3] The level of indomethacin was that which would inhibit repair without inducing gastric mucosal ulceration and the misoprostol was given in a dose which would reverse this effect. Prednisolone was given in a dosage shown to modulate ribosomal RNA synthesis in rats.[4]

On days 3 and 6 after ulcer induction, four animals from each group were given an intraperitoneal injection of BrdU (100 μg/g body weight). They were killed an hour later for determination of ulcer size and proliferation activity assessment at ulcer edges.

ULCER INDUCTION

Ulcers were induced as described[3 5] in unfasted rats after laparotomy performed under anaesthesia by intramuscular injection of 1 ml/kg Hypnorm (fentanyl citrate 0·315 mg/ml, fluanisone 10 mg/ml, Janssen Pharmaceuticals) at 9.00 am. The cryoprobe was cooled to equilibrium in liquid nitrogen and then applied to the serosal surface of the anterior wall of the stomach for 45 seconds at two different sites 8 mm apart. After recovery animals were allowed free access to rat chow and water.

ULCER SIZE ASSESSMENT

After death the rat's stomach was opened and the ulcer size was determined in the pinned out stomach by square counting under a dissecting microscope.

ASSESSMENT OF PROLIFERATIVE ACTIVITY AT ULCER EDGES

Gastric ulcers with surrounding mucosa were fixed in formaldehyde (4%) for immunostaining with PCNA or Carnoy's solution for immunostaining with anti-BrdU, and mounted in paraffin. Sections (4 μ) were then ready for immunostaining.

BrdU IMMUNOHISTOCHEMISTRY

Tissue from animals that had been injected with BrdU one hour before death was fixed in Carnoy's and mounted as described above. Sections were deparaffinised in xylene. The first layer antibody was a mouse IgG monoclonal anti-BrdU antibody and after a further conventional peroxidase antiperoxidase method, sections were incubated with diaminobenzidine with 0·01% H_2O_2. Tissue was then counterstained with haematoxylin, rehydrated, and mounted in Pertex solution. For both BRDU and PCNA staining, irrelevant first layer and buffered saline controls were performed.

PCNA IMMUNOHISTOCHEMISTRY

Formaldehyde fixed tissue was cut into sections (4 μ) and mounted on poly L-lysine glass slides. Sections were then dewaxed and immunostaining was performed using the ABC method with primary incubation with PCNA overnight. Diaminobenzidine-hydrogen peroxide was employed as a chromogen. Tissue was counterstained with haematoxylin and mounted in Pertex solution.

PROLIFERATIVE ACTIVITY ANALYSIS

Well oriented glands were counted from the biopsy specimens for quantitation of proliferative activity. In every ulcer, 20 contiguous gastric glands adjacent to the ulcer edge were identified and proliferating cells were counted under a microscope.

STATISTICAL ANALYSIS

Ulcer size was measured in mm^2 and data are presented as mean (SEM). Cell proliferation values are presented as number of labelled cells (mean (SD)). Unpaired Student's *t* tests were used in the analysis of ulcer size. The relation between results obtained with BrdU and PCNA labelling was assessed with linear regression analysis.

Results

A similar weight gain was observed in rats in all five treatment groups.

ULCER SIZE

On day 3 the area of mucosal ulceration in control animals was 3·1 (0·4) mm^2. The ulcer area was significantly larger in animals treated with indomethacin (4·8 (0·3) mm^2; $p<0·05$) and prednisolone (5·7 (1) mm^2; $p<0·05$) than in controls. The ulcer size in animals that received misoprostol plus prednisolone was 2·5 (0·8) mm^2, a value not significantly different from controls but significantly smaller than

TABLE I *Area of surface ulceration in gastric mucosae at the site of cryoprobe three and six days after ulcer induction*

	Mean (SEM) ulcer size (mm²)	
Treatment groups	*At 3 d*	*At 6 d*
Control	3·1 (0·4)	1·6 (0·3)
Indomethacin	4·8 (0·3)*	3·1 (0·2)*
Prednisolone	5·7 (1·0)*	3·4 (0·3)*
Prednisolone+misoprostol	2·5 (0·8)†	2·0 (0·0)†
Misoprostol	2·2 (0·3)†	1·6 (0·2)†

*p<0·05 *v* control; †p<0·05 *v* Prednisolone.

that in the group treated with prednisolone alone (5·7 (1) mm²; p<0·05). Ulcer size in animals treated with misoprostol alone was not significantly different from control animals (2·2 (0·3) mm² vs 3·1 (0·4) mm²). Similar results for all these between group comparisons were obtained after 6 days (Table I). As summarised in Table I, ulcers in both prednisolone and indomethacin treated rats were larger than those in control animals, and coadministration of misoprostol with corticosteroids returned the ulcer size to that seen in the control group.

CELL PROLIFERATION RATE ANALYSIS

Assessment of cell proliferation at the ulcer edges was performed by immunohistochemical localisation of PCNA and BrdUrd on day 3 (Table II).

BrdU localisation

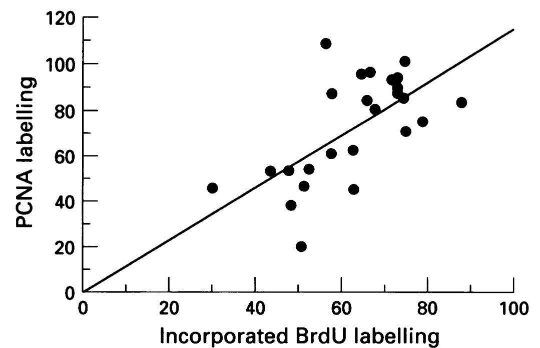
In rats treated with prednisolone and indomethacin there were fewer immunopositive cells per 20 gastric crypts (49 (9); p<0·02 and 53 (15); p<0·02, respectively) than in the control group (70 (9·9)). Values in animals treated with misoprostol compared with controls were (73 (5) *v* 70 (9·9); p=NS). The number of positively stained cells in animals treated with prednisolone and misoprostol (68 (8)) did not differ from controls.

PCNA localisation

Immunostaining with PCNA also showed a decreased number of immunopositive cells in rats treated with prednisolone and indomethacin (52 (7); p<0·0001 and 43 (7·7); p<0·0001, respectively) compared with controls (96 (10)). Animals treated with prednisolone plus misoprostol showed fewer immunopositive cells (88 (6·5)) but this value was not significantly different from controls. The number of proliferating cells in the group treated with misoprostol alone (80 (4·7)) was

TABLE II *Results of immunostaining with proliferating cell nuclear antigen (PCNA) and incorporated 5-bromo-5-iododeoxyuridine (BrdU) three days after ulcer induction in controls and treatment groups (values mean (SD))*

Treatment group	*PCNA*	*BrdU*
Control	96 (10)	70 (9)
Indomethacin	43 (7·7)	53 (15)
Prednisolone	52 (7)	49 (9)
Prednisolone+misoprostol	88 (6·5)	68 (8)
Misoprostol	80 (4·7)	73 (5)



Correlation between cell numbers measured by incorporated 5-bromo-5-iododeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA) immunolabelling techniques, three days after ulcer induction (r=0·64; p<0·001).

not significantly different to that in the control group.

Comparison between BrdU and PCNA immunolabelling.

Both immunohistochemistry techniques showed that the proliferative rate in gastric crypts in the healing ulcer edge is inhibited by treatment with prednisolone and indomethacin, and that in prednisolone treated animals coadministration of misoprostol returns the rate to that seen in the control group. Regression analysis showed a significant correlation between the two immunolabelling techniques (r=0·64; p<0·001) (Figure).

Discussion

Our results show that experimental ulcers induced by cryoprobe are larger and slower to heal in rats given prednisolone. This observation, if parallelled in man, could indicate a mechanism for some of the epidemiological features of gastrointestinal damage during corticosteroid treatment. Whatever initiates the ulceration, a consequence of delayed healing would be that ulcers persist for longer, and thus would seem more prevalent compared with controls: furthermore if ulcers take longer to heal, each episode of ulceration might have a greater chance of leading to haemorrhage.

Our observations on delayed healing of experimental ulcers during prednisolone therapy agree with the work of Kuwayama *et al* who also showed that hydrocortisone slowed healing of acetic acid induced ulcers in animals.[6 7] In investigating the mechanism of delay in healing, they showed that cell renewal in *normal* gastric mucosa was delayed by hydrocortisone. That finding was parallelled in man by Eastwood *et al.*[8 9] They studied gastric fundic mucosa in healthy volunteers before and after two weeks' treatment with prednisolone and reported an inhibition of epithelial cell proliferation in human fundic mucosa. There are at least two mechanisms by which an epithelium with a depressed turnover might be slower to heal. The first involves cell motility – the presence of older, potentially less mobile mucosal cells might lead to a failure of mucosal restitution, independent of cell replication. The second mechanism involves

an alteration in the proliferative response to injury. The study reported here shows that the delay in healing induced by corticosteroids involved a diminution in the processes of cell proliferation that causes regenerative repair at the ulcer edge, with a reduction in DNA synthesis in the proliferative zone of gastric crypts adjacent to the ulcers.

The mechanisms by which corticosteroids affect gastric cell proliferation are not fully understood but an effect via prostaglandin metabolism seems likely. Corticosteroids inhibit phospholipase A_2, which influences the liberation of arachidonic acid, the first step in the generation of prostaglandins. Corticosteroids also stimulate the production of a number of proteins that inhibit prostanoid action, such as lipocortin.[10 11] Eastwood *et al* showed an inhibitory effect of hydrocortisone sodium succinate on prostaglandin generation in the rat fundic mucosa.[12] Exogenous PGE_2 and PGE_1 reversed the effects of hydrocortisone in delaying in healing of acetic acid induced ulcers.[7] Our results show that PGE_1 reversed the effect of prednisone on cryo-ulcers, and, in terms of regenerative repair, the effect of the corticosteroid on ulcer healing is similar to that of the prostaglandin synthetase inhibitor indomethacin.

In addition to effects on regenerative repair, inhibition of prostaglandin synthesis by corticosteroids will have an effect on a number of other prostaglandin mediated defence mechanisms: thickening of gastric mucus, stimulation of active bicarbonate secretion, changes in vascular supply, acid inhibition, increased flux of water from the serosa to the mucosa, and possibly inhibition of adhesion of neutrophils to blood vessels.[13]

Many of these prostaglandin mediated cytoprotective effects and the inhibitory effects of corticosteroids on prostanoids have been demonstrated more readily in animals than man, and the relevance that each of these effects has to ulcer healing in the clinical situation remains unclear. With regard to the dose of corticosteroid used in our study, and in similar experiments,[6 7] this is higher than that normally used long term treatment (0·1–1 mg/kg) but similar to that used short term in conditions such as graft rejection (10–15 mg/kg). As already mentioned, the clinical association between corticosteroid therapy and peptic ulcer is best established at higher dosage.[1]

In this study we compared immunostaining for incorporated BrdU with staining for PCNA. Antibodies to PCNA have proved to be effective in quantitatively assessing nuclear labelling in a variety of tissues including the colon and the liver; this study shows their value in quantitating cell proliferation in rat gastric mucosa and confirms that the technique provides similar results to those obtained with BRDU staining after in vivo labelling.[14 15] The technical advantages of the PCNA technique, giving spatial and quantitative assessment of proliferation as does the BrdU technique, but without in vivo injections of label, and with the advantages of applicability to paraffin embedded tissue, make this a highly suitable marker for assessing regeneration in experimental ulcer disease.[16 17]

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