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Prostaglandin, tumor necrosis factor α and neutrophils: causative relationship in indomethacin-induced stomach injuries

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Abstract

Tumor necrosis factor alpha (TNF-α) has been suggested to play a critical role in indomethacin-induced gastric mucosal damage, so we evaluated its mucosal level and its relationship with prostaglandin E_2 and neutrophils in indomethacin-induced gastric mucosal injury in rats. Indomethacin caused a time- and dose-dependent increase in gastric mucosal erosion, which was accompanied by a reduction in prostaglandin E_2 followed by an increase in TNF-α level and neutrophil infiltration in the gastric mucosa. Pretreatment with exogenous prostaglandin E_2 totally abolished indomethacin-induced gastric mucosal injury and the TNF-α increase. Depletion of neutrophils by methotrexate or reduction of TNF-α concentration by pentoxifylline markedly reduced indomethacin-induced mucosal damage. Pentoxifylline but not methotrexate prevented the increase in mucosal TNF-α level induced by indomethacin. It is suggested that depletion of prostaglandin E_2 followed by an increase of TNF-α production and neutrophil infiltration in the gastric mucosa are important sequential processes in indomethacin-induced ulceration. Prevention of one of these processes would inhibit ulcer formation.

Keywords: Indomethacin; Prostaglandin; TNF-α (tumor necrosis factor-α); Neutrophil; Gastric ulceration

1. Introduction

Gastrointestinal damage and bleeding are the major side effects of non-steroidal anti-inflammatory drugs (NSAID). These side effects seriously affect their clinical applications. Moreover, the mechanisms of this ulcerogenic action are not fully understood. Previous studies have suggested that depletion of prostaglandin, neutrophil accumulation, impairment of mucosal blood flow and reduction of mucosal cell proliferation all contribute to the pathogenic mechanisms (Scarpignato, 1995; Wallace, 1992). Recently, increasing evidence has indicated that the neutrophil–endothelial cell interaction is a critical and early event in the pathogenesis of NSAID-induced gastric injury (Yoshida et al., 1993; Wallace et al., 1993). Indeed, use of specific anti-neutrophil antibody or methotrexate to cause neutropenia, interference with neutrophil adhesion to the endothelium of blood vessels, or use of monoclonal antibodies against neutrophil CD11b/CD18 expression significantly reduces gastric mucosal injury by NSAID (Alican et al., 1995; Wallace et al., 1990, 1991).

In vitro, proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-1β, and interferon-γ have been suggested to potentiate intercellular adhesion molecule (ICAM-1) expression on the endothelium (Eissner et al., 1994), to favour neutrophil adhesion and cause mucosal damage. Furthermore, the local TNF-α level has been suggested to be critical in tissue and organ damage during the early phase of neutrophil-mediated inflammation (Hashimoto et al., 1994; Von Asmuth et al., 1991). Reports from other studies (Santucci et al., 1994, 1995a,b; Appleyard et al., 1996) indicated that there was an increased peripheral blood TNF-α level in indomethacin-challenged rats, which suggests that TNF-α could play an important role in indomethacin-induced gastric mucosal injury. The role of TNF-α in mediating NSAID-induced gastric damage was further supported by the finding that reduction of plasma TNF-α concentration by pentoxi-

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fylline, dexamethasone, or TNF-α antibody could also reduce gastric mucosal damage. However, the mucosal level of TNF-α, which is directly linked to gastric inflammation, has not been determined, and how this cytokine correlates with mucosal neutrophil infiltration is still undefined. Therefore, clarification of the sequential changes associate with ulcerogenic processes in gastric tissue is important to obtain a full understanding of the pathogenesis of indomethacin-induced ulceration.

Numerous studies have indicated that exogenous prostaglandin E_2 or its analogues can attenuate indomethacin- or aspirin-induced gastric mucosal damage, but its protective mechanisms are not fully understood. Exogenous administration of prostaglandin E_2 reduces both plasma TNF-α level and attenuates the gastric mucosal damage elicited by indomethacin (Appleyard et al., 1996), suggesting that the protective action of prostaglandin E_2 might be exerted through inhibition of plasma TNF-α levels. While the plasma TNF-α level reflects a general pathological condition in the animal, it does not directly represent the inflammatory changes in the gastric mucosa. Furthermore, the relationship between mucosal prostaglandin E_2 depletion and TNF-α release after indomethacin administration is currently unknown. In the present study, we aimed to determine the sequential relationship between prostaglandin E_2 depletion and mucosal TNF-α level in indomethacin-induced mucosal damage. In addition, we also used neutrophil modulators to study how neutrophils interact and relate to TNF-α and ulcer formation after indomethacin administration, in order to define the causative relationship among these substances.

2. Material and methods

2.1. Animal and reagents

Male Sprague–Dawley rats, weighing 180–220 g and obtained from the Animal Unit, the University of Hong Kong, were used in our study. Animals were fasted for 24 h, but allowed free access to tap water before experimentation. All chemicals and antibodies used in this study were purchased from Sigma unless specified.

2.2. Mucosal erosion evaluation

Animals ($n = 10–12$) were injected subcutaneously with indomethacin at doses of 5 mg/kg or 30 mg/kg. The drug was prepared in a solution of 10% ethanol and 15% Tween-80. Control animals received the same solution without indomethacin. All reagents were freshly prepared 1 h before the experiments. Animals were killed at 2, 4, 6 or 8 h after drug treatment. The stomach was quickly removed and opened along the greater curvature. The mucosal damage was evaluated by a single blinded approach (observer was unaware of the treatment groups) with a dissecting microscope. The haemorrhagic lesions were recorded and expressed as length in millimeter. As soon as the mucosal erosions were recorded, the gastric mucosal layer was scraped off at 4°C and quickly frozen in liquid nitrogen and transferred to −70°C until assayed. In separate experiments, the actions of pentoxifylline, methotrexate and prostaglandin E_2 on indomethacin-induced mucosal damage were examined 6 h after drug administration, when mucosal damage was most evident. Pentoxifylline (200 mg/kg) and prostaglandin E_2 (1 mg/kg) were injected intraperitoneally into rats 2 h after indomethacin administration. Methotrexate (2.5 mg/kg) was given once a day for 5 days before the rats were used.

2.3. Mucosal prostaglandin E_2 assay

We used a commercially available radioimmunoassay (RIA) kit (Amersham, UK). Briefly, rat gastric mucosal tissues were weighed and homogenized at 4°C in a Krebs solution for 30 s and centrifuged at 12 000 rpm (Beckman J2-21) for 20 min at 4°C. Supernatants were used for the prostaglandin E_2 assay. The prostaglandin E_2 levels were then assayed with a RIA kit, following the manufacturer's protocol. Protein was determined by a modified Lowry's method (Read and Northcote, 1981), and the prostaglandin E_2 concentrations were expressed as pg per mg protein.

2.4. Determination of mucosal TNF-α level

Mucosal TNF-α level was assayed by using a commercially available TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Genzyme Diagnostics, Cambridge, USA). We chose a mouse TNF-α ELISA kit, which was cross-reacted with rat TNF-α. Briefly, gastric mucosal samples kept at −70°C were weighed and homogenized, after thawing, in 100 mg sample/0.9 ml phosphate buffer saline solution (PBS pH 7.2) at 4°C for 20 s. They were centrifuged at 12 000 rpm (Beckman J2-21) for 20 min. One hundred microlitres of the supernatants were added to the wells of 96-well microplates precoated with mouse anti-TNF-α antibody and incubated at 37°C for 2 h. After being washed 4 times with PBS, horseradish peroxidase-conjugated goat anti-mouse TNF-α antibody was added and incubated for 1 h at 37°C. After the walls were washed four times, the substrate solution was added. The reaction took place in the dark for 10 min at room temperature, and was stopped with 2 M H_2SO_4 solution. The samples were read in a microplate reader (Bio-Red, Modle 3550, USA) at 450 nm within 30 min. Protein concentration in the sample was determined by a modification of Lowry's method as mentioned above. The TNF-α value was expressed as pg per mg protein. Inter-batch and intra-batch variations were less than 8% and 4%, respectively.

Pentoxifylline was used to determine its effects on mucosal TNF-α levels. Pentoxifylline at 200 mg/kg, the most effective dose for reducing the peripheral blood

TNF-α level (Santucci et al., 1994, 1995b), was given intraperitoneally to rats 2 h after indomethacin administration. Rat gastric mucosa was prepared as stated above. To observe the influence of neutrophils on TNF-α and on gastric damage, rats were given methotrexate 2.5 mg/kg intraperitoneally, once a day for 5 days, a known dose to effectively reduce neutrophil number (Alican et al., 1995). Before and after methotrexate injection, smears from tail vein blood were made and stained with hematoxylin and eosin to count total blood leukocyte number and to estimate the neutrophil percentage.

Prostaglandin E_2 at the dose of 1 mg/kg was administered intragastrically 2 h after indomethacin administration to observe its protective action on gastric mucosa, and its effect on the mucosal TNF-α level. The gastric mucosal preparation and the mucosal damage evaluation were the same as stated above.

2.5. Peripheral blood neutrophil percentage and mucosal neutrophil counts

Rats were anaesthetized with ether, two drops of tail vein blood were collected and fixed with 96% ethanol and stained with hematoxylin and eosin. At least 400 leukocytes were counted in 2 smears to determine neutrophil and monocyte percentages in the total leukocyte count. To evaluate mucosal neutrophil infiltration, animals were divided into 6 different time groups of 0.5, 1, 2, 4, 6, 8 h after indomethacin injection, or its vehicle. The gastric specimens (corpus) were collected as stated above and fixed in 10% formalin–PBS solution overnight, and then the gastric samples were processed by routine paraffin-embedding procedures. Six-micrometre sections were prepared and stained with hematoxylin and eosin. The mucosal neutrophil numbers were assessed by two pathologists who were blinded from the treatment groups. The neutrophil number was counted in the same number of fields around the blood vessel and non-blood vessel areas in the mucosa of each section ($n = 5$). At least 10 fields were evaluated to yield an average neutrophil number in each sample.

2.6. Statistical analysis

All values are expressed as means $\pm$ S.E. Data were analysed by using Student's *t*-test; a P value less than 5% was considered as significant. The relationship between mucosal damage, TNF-α, and peripheral blood neutrophil accumulation was evaluated by the linear correlation analysis.

3. Results

3.1. Gastric mucosal injury

Indomethacin administration induced a time- and dose-dependent increase in gastric mucosal injury (Fig. 1).

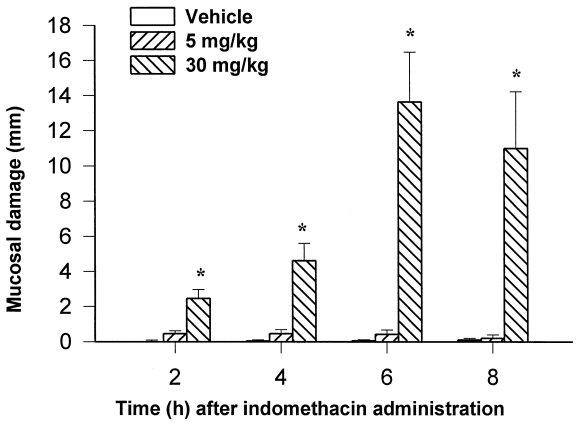

Fig. 1. Effects of indomethacin on gastric mucosal damage. Indomethacin (5 or 30 mg/kg) was given subcutaneously at zero hour, and the rats were killed at different times. Values are the means for 10–12 rats, vertical bars represent S.E., $* P < 0.005$ compared with the control and 5 mg/kg indomethacin groups.

Significant mucosal erosion was observed in the 30 mg/kg dose group, beginning at 2 h and reaching the peak at 6 h after injection. There was a slight decrease in ulcer size at

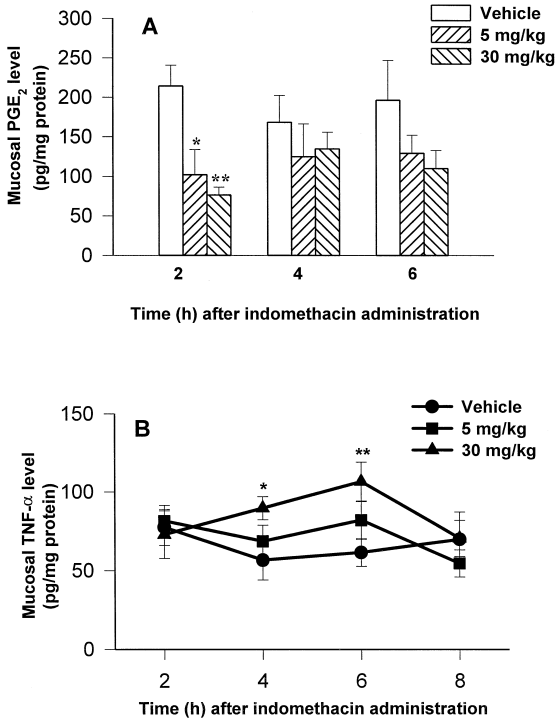

Fig. 2. Effects of indomethacin on mucosal prostaglandin E_2 (A) and NF-α levels (B). Rats were injected subcutaneously with vehicle or indomethacin (5 mg/kg or 30 mg/kg) and killed 6 h later. The mucosal prostaglandin (PGE_2) level was measured by radioimmunoassay, and the NF-α level was measured by ELISA. Values are the means for 6 rats, vertical bars represent S.E., $* P < 0.05$, $** P < 0.01$ compared with the control.

8 h after drug administration. The vehicle-injected group showed no visible mucosal erosion.

3.2. Mucosal prostaglandin E_2 and TNF-α level

Fig. 2A shows the gastric mucosal prostaglandins E_2 levels after indomethacin challenge. The prostaglandins E_2 level was significantly inhibited by indomethacin administration at 2 h in both 5 mg/kg and 30 mg/kg groups when compared with that of the respective vehicle group, but showed no significant difference at 4 and 6 h after indomethacin administration.

The changes of mucosal TNF-α concentrations after indomethacin challenge are shown in Fig. 2B. The mucosal TNF-α concentrations at 4 and 6 h in the 30 mg/kg indomethacin administration group were significantly increased when compared with those of the vehicle group, while in the 5 mg/kg group, TNF-α concentrations did not increase significantly over the control at any time. At 8 h, the TNF-α value was not significantly different between the vehicle control and the indomethacin-treated groups. The increase in TNF-α occurred 2 h after the mucosal prostaglandin E_2 changes.

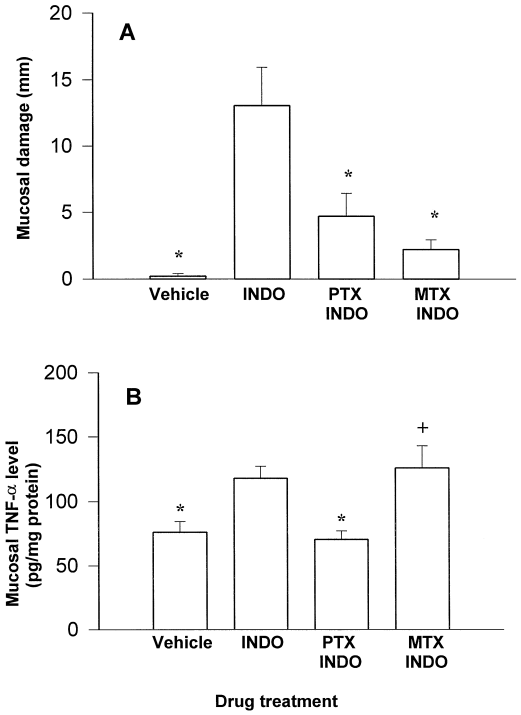

Fig. 3. Effects of pentoxifylline or methotrexate on indomethacin-induced gastric mucosal damage (A) and TNF-α level (B). Rats were given subcutaneously indomethacin (INDO, 30 mg/kg) and killed 6 h later. Pentoxifylline (PTX, 200 mg/kg) was given 2 h after indomethacin administration. Methotrexate (MTX, 2.5 mg/kg) was injected once a day for 5 days before the experiment. Values are the means for 6–8 rats, vertical bars represent S.E., $* P < 0.05$, $+ P > 0.05$ when compared with the INDO alone-treated group.

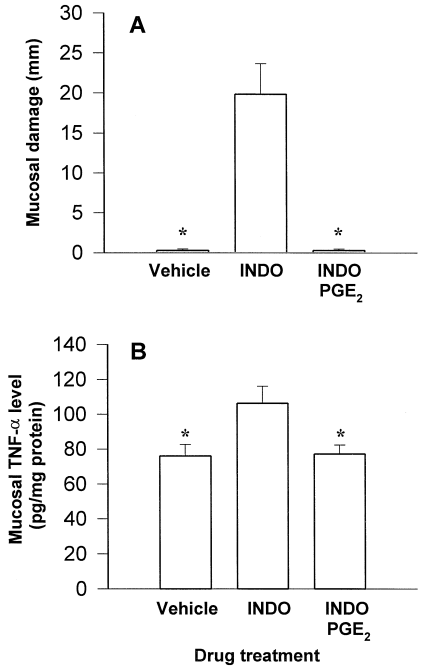

Fig. 4. Effects of exogenous prostaglandin E_2 administration on indomethacin-induced gastric mucosal damage (A) and TNF-α changes (B). Rats were injected subcutaneously with indomethacin (INDO, 30 mg/kg) and killed 6 h later. Prostaglandin E_2 (PGE_2, 1 mg/kg) was given orally 2 h after INDO administration. Values are the means for 6–7 rats, vertical bars represent S.E., $* P < 0.05$ when compared with the INDO alone-treated group.

3.3. Effects of pentoxifylline and methotrexate on mucosal erosion and TNF-α level

Six hours after indomethacin challenge, the rat gastric mucosal damage index and TNF-α level were significantly increased over those of the control group. Pentoxifylline and methotrexate markedly prevented this mucosal damage by 64% and 83.1%, respectively (Fig. 3A). The increased TNF-α level was largely abolished by pentoxifylline, while methotrexate injection did not influence the mucosal TNF-α level compared with that of the indomethacin alone-treated groups (Fig. 3B).

3.4. Effects of prostaglandin E_2 in mucosal damage and TNF-α level

Oral administration of 1 mg/kg prostaglandin E_2 to rats completely abolished indomethacin-induced gastric mucosal injury at 6 h after injection (Fig. 4A). Prostaglandin E_2 pretreatment also abolished the indomethacin-induced TNF-α increase in the gastric mucosa (Fig. 4B).

3.5. Peripheral blood neutrophil counts

The percentage of neutrophils in the blood of the 30 mg/kg indomethacin group was high at all times, but reached statistical significance only at 6 h after indo-

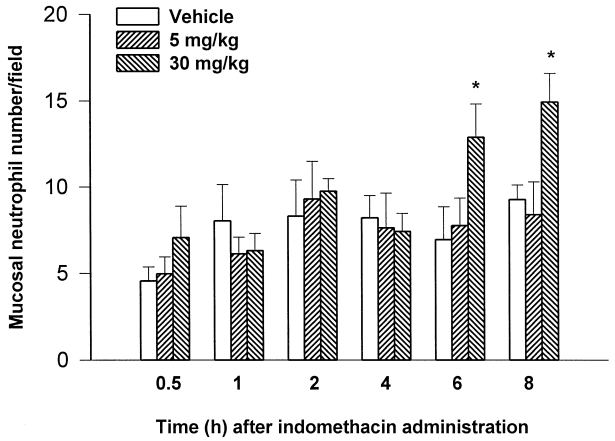

Fig. 5. Effects of indomethacin on gastric mucosal neutrophil number. Rats were injected subcutaneously with indomethacin (30 mg/kg) and killed 0.5, 1, 2, 4, 6 or 8 h later. Values are the means for 5 rats, vertical bars represent S.E., $* P < 0.05$ when compared with the respective vehicle-injected control.

methacin administration ($40.71 \pm 3.58\%$ vs. $20.82 \pm 3.37\%$ for the control, $P < 0.05$). Methotrexate injection significantly decreased the total blood leukocyte number (6029 ± 954 mm^3 vs. $10\,180 \pm 695$ mm^3 for the control, $P < 0.01$), as well as the neutrophil percentage ($8.5 \pm 1.21\%$ vs. $15.48 \pm 2.64\%$ for the control, $P < 0.05$). In all the samples counted, the monocyte percentage remained the same in the drug-treated and control groups (data not shown).

3.6. Mucosal neutrophil infiltration

Mucosal neutrophil infiltration was significantly increased in the 30 mg/kg group at 6 and 8 h after indomethacin administration, compared with that of the control groups (Fig. 5), whereas at 0.5, 1, 2 and 4 h the neutrophil numbers were not significantly different from those of the control group. This infiltration was also significantly correlated with mucosal damage and time after indomethacin administration when analysed by linear correlation ($r = 0.827$, 0.879, $P < 0.05$). In the 5 mg/kg indomethacin dose, there was no significant increase in neutrophil infiltration over the control in all time groups.

4. Discussion

Previous studies have shown that NSAID-induced gastric mucosal damage is attenuated by prior supplementation with prostaglandin or depletion of circulatory neutrophils by antiserum or methotrexate (Alican et al., 1995; Wallace et al., 1990, 1991, 1993). In addition, indomethacin also increase the concentration of TNF-α, a pro-inflammatory cytokine in the blood, which suggests that not only prostaglandin and neutrophils are involved in ulcer formation, but that TNF-α could also play a significant role in NSAID-induced gastric mucosal injury. However, how these substances interact and modulate the ulcerogenic processes during ulcer formation are still undefined. In order to delineate the interrelationship of these substances in indomethacin ulceration, the mucosal levels of these mediators should be measured in a sequential manner, so that the ulcerogenic processes provoked by NSAID can be better defined. We therefore studied the mucosal concentrations of prostaglandin E_2, TNF-α and the number neutrophils at different times after indomethacin administration.

In the present study, the mucosal concentration of prostaglandin E_2 was significantly reduced in the first 2 h after indomethacin challenge, especially after the higher dose. Concentrations were restored at 4 and 6 h after drug administration. This result was in accord with another observation in which the autacoid was similarly reduced during acute jejunum injury (Nygard et al., 1994). This decrease could initiate the subsequent ulcerogenic processes in the gastric mucosa. Indeed, inhibition of prostaglandin E_2 synthesis in the mucosa was reported to be accompanied by early gastrointestinal microvascular damages and smooth muscle change (Nygard et al., 1994; Tarnawski et al., 1990). These events could trigger the production of inflammatory cytokines, which further damage the gastric mucosa.

Prostaglandin E_2 has a modulatory role on TNF-α production and is also a potent inhibitor of neutrophil adherence and chemotaxis (Watanabe et al., 1994). It has recently been hypothesized that inhibition of neutrophil adherence by prostaglandin activation is one of the possible anti-ulcer mechanisms against NSAID-induced gastrointestinal damage (Wallace, 1992). This idea was supported by the findings of Asako and his associates that both misoprostol and prostacyclin were able to block indomethacin- or aspirin-induced rat leukocyte adherence to the mesenteric venules via an unknown mechanism (Asako et al., 1992a,b). Furthermore, our study also demonstrated for the first time that prostaglandin E_2 inhibited mucosal TNF-α production and simultaneously reduced indomethacin-induced gastric mucosal damage. This finding is also in line with previous observations, indicating that rat gastric mucosal damage and increase in plasma TNF-α can be prevented by pretreatment with prostaglandin E_2 or dexamethasone (Appleyard et al., 1996). These findings would explain, in part, the role of prostaglandin in the modulation of TNF-α levels in both the blood and the gastric mucosa. The relationship between prostaglandin and TNF-α is further strengthened by the results of in vitro studies. Exogenous prostaglandin E_2 dose dependently suppressed TNF-α release and its expression in human blood monocytes and the effects of indomethacin on TNF-α release and expression were due to the inhibition of endogenous prostaglandin E_2 production (Spatafora et al., 1991). Based on these findings and the observations of the present study, it is likely that prostaglandin inhibits the stimulatory effect of indo-

methacin on TNF-α release in the gastric mucosa. This effect could lead to a reduction in neutrophil activation and subsequently decreased ischemia and mucosal damage.

The pathogenic effects of TNF-α in indomethacin-induced gastric mucosal damage still remain elusive, but enhancement of the neutrophil–endothelial interaction is supposed to be one of its ulcerogenic mechanisms. In fact, neutrophil adhesion to the vascular endothelium requires the expression of neutrophil CD11b/CD18 and endothelial ICAM-1. Both indomethacin and TNF-α are able to stimulate ICAM-1 expression, but only TNF-α has been shown to potentiate neutrophil CD 18 synthesis (Gamble et al., 1985; Limb et al., 1991; Walsh et al., 1991). Inhibition of TNF-α by prostaglandin E_2 could result either directly or indirectly in the reduction of neutrophil CD11b/CD18 and endothelial ICAM-1 expression, which subsequently reduces neutrophil adhesion in the blood vessels. Further studies are required to identify the mechanisms by which TNF-α modulates neutrophil CD11b/CD18 expression during indomethacin-induced ulceration in the gastric mucosa.

In vitro investigation has shown that indomethacin increases the expression of mRNA for TNF in peripheral blood mononuclear cells 1 h after incubation (Tsuboi et al., 1995). The increase in plasma TNF-α could also be detected as early as 30 min after indomethacin administration (Appleyard et al., 1996). In the present study, the mucosal TNF-α concentration was elevated 4 and 6 h after indomethacin administration, suggesting that the early increase in plasma TNF-α at 30 min after indomethacin injection was unlikely to be due to TNF-α from gastric mucosa. The mucosal increase in TNF-α level was paralleled by the mucosal damage in the stomach but not strictly related to peripheral and mucosal neutrophil accumulation. The fact that depletion of neutrophils by methotrexate did not reduce the mucosal TNF-α level compared with control suggests that neutrophils are not the major source of TNF-α in this model. Instead, it has been shown that both monocytes and macrophages are important sources for this cytokine during acute inflammation (Schreiber et al., 1993; Yamakawa et al., 1995). It is hypothesised that both local resident and systemic macrophages or immunocytes might contribute to the local increase in TNF-α concentration.

The observation, that indomethacin stepwisely increased neutrophil infiltration at 6 and 8 h after its administration, while the level of TNF-α was found to decrease and return to normal 8 h after injection, further supports the above sequential phenomenon. It is envisaged that neutrophil accumulation in the gastric mucosa occurs after TNF-α stimulation, which in turn occurs between 4 and 6 h after indomethacin injection. The mechanism for neutrophil infiltration in the gastric mucosa is currently not known, and a direct effect of TNF-α on neutrophil infiltration has not been reported. It is possible that indomethacin could indirectly trigger the release of cytokine, which could induce neutrophil aggregation in the gastric mucosa. Indeed, TNF-α has been demonstrated to induce interleukin-8 production from a variety of cell types, including gastric epithelial cells, immunocytes and endothelial cells. This cytokine is a potent chemoattract mainly for neutrophils (Yasumoto et al., 1992).

The importance of TNF-α stimulation and neutrophil infiltration in the pathogenesis of indomethacin ulceration was also confirmed in the present study. The decrease in TNF-α concentration by pentoxifylline, and the depletion of neutrophils by methotrexate both reduced the ulcerogenic action of indomethacin. As methotrexate showed no influence on the indomethacin-induced increase in mucosal TNF-α over the control level, the protection exerted by methotrexate is probably beyond its action on this cytokine, and this also further supports that neutrophil infiltration occurred after TNF-α stimulation. Indeed, the drug not only reduces the number of circulating neutrophil, but also attenuates the action of platelet-activating factor on leukocyte-endothelial cell adhesion in postcapillary venules (Asako et al., 1993). All these effects could account for the protective action of methotrexate in the later stage of indomethacin-induced ulceration.

In summary, indomethacin induced a time- and dose-dependent increase in gastric mucosal damage in rats, which peaked at 6 h after drug administration. We suggest that early depletion of prostaglandin in the gastric mucosa in the first 2 h could lead to subsequent inflammatory changes in the gastric mucosa, including an increase in TNF-α in the tissue followed by neutrophil accumulation in the later stage of ulceration. Prevention of either one of these processes by prostaglandin E_2, pentoxifylline or methotrexate could inhibit indomethacin-induced ulceration of the gastric mucosa.

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