Annexin-1 modulates repair of gastric mucosal injury

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Annexin-1 modulates repair of gastric mucosal injury. Am J Physiol Gastrointest Liver Physiol 294: G764–G769, 2008. First published January 17, 2008; doi:10.1152/ajpgi.00531.2007.—Annexin-1 is a glucocorticoid-inducible protein that plays an important effector role in the resolution of inflammation and has recently been shown to contribute to the resistance of the stomach to injury. Using an integrated genetic and pharmacological approach, we have tested the hypothesis that annexin-1 contributes to the healing of mucosal injury, given that such injury is accompanied by an inflammatory response, which is often associated with an overexpression of annexin-1 expression. Gastric ulcers were induced in mice through serosal application of acetic acid. Annexin-1 expression during the healing of the ulcers was examined. The effects on gastric ulcer healing of treatment with an annexin-1 mimetic (Ac2-26), an antagonist of the annexin-1 receptor (Boc2), or a glucocorticoid (dexamethasone) were examined. Finally, susceptibility to and healing of indomethacin-induced gastric lesions were compared in wild-type and annexin-1-deficient mice. Expression of annexin-1 was significantly increased in the gastric ulcer margin throughout the healing process. Treatment with an annexin-1 mimetic (Ac2-26) significantly enhanced gastric ulcer healing. In contrast, both dexamethasone and an formyl peptide receptor-like-1 (FPRL-1) antagonist impaired the early phase of ulcer healing. Annexin-1-deficient mice exhibited the same susceptibility as wild-type mice to indomethacin-induced gastric damage, but the healing of that damage was impaired in the former. These data support the hypothesis that annexin-1 contributes significantly to the process of healing of gastric mucosal damage.

MATERIALS AND METHODS

Animals. Animal studies were conducted in accordance with the guidelines established by the Canadian Council of Animal Care, and all protocols were approved by the Animal Care Committee at the University of Calgary. Male C57BL/6 mice (25–27 g) were fed standard laboratory chow and water ad libitum. The mice were housed in pairs and kept in a room with controlled temperature (22 ± 1°C), humidity (65–70%), and light cycle (12 h:12 h light-dark).

Induction of gastric ulcers. Ulcers were induced by using a modification of a previously described model (9). In brief, mice were anesthetized with halothane, the abdomen was opened with a midline laparotomy, and the stomach was exposed to permit application of glacial acetic acid (200 μl, 20% vol/vol) to the serosal surface of the corpus region. This was applied for 1 min via a 1-ml syringe barrel (28.2-mm2 contact area). The acetic acid was then removed, and the area of contact was washed three times with 200 μl of saline. The peritoneum was closed with interrupted 5–0 silk sutures, and the skin was closed with a running suture of 4–0 vicryl.

Measurement of ulcer area. Subgroups of mice were euthanized by cervical dislocation, and their stomachs were removed and opened with an incision along the greater curvature. The stomach was then pinned out, mucosal side up, on a paraffin block flooded with ice-cold saline. A 25-mm2 grid was placed alongside the ulcer, and digital photographs were taken. The area of ulceration (mm2) was blindly measured by planimetry.

Drug treatments. Ac2-26 (acetyl-AMVSEFLKQAWIENEEQY- VVQTVK) is a synthetic peptide that exerts many of the anti-inflammatory and protective actions of annexin-1 (14, 15). The dose of Ac2-26 was selected based upon previous published results in which the treatment of mice with the annexin-1 mimetic was shown to significantly attenuate both polymorphonuclear neutrophil (PMN) inflammation and cell proliferation, formation of granula- tor-like-1 (FPRL-1) (2, 10, 14). However, annexin-1 expression can also be regulated independent of glucocorticoids (26, 29). The ability of annexin-1 to interfere with lymphocyte and monocyte migration and to induce neutrophil apoptosis is considered important in the anti-inflammatory effects of this protein (12, 17, 20).

Little is known of the potential contributions of annexin-1 to the process of healing in the gastric mucosa. However, a recent study suggests that annexin-1 may contribute to the ability of the rat stomach to resist injury (34). Given the apparent contradictory findings that annexin-1 contributes to mucosal defense, but glucocorticoids, which can modulate annexin-1 expression, impair repair of mucosal injury, the present study was performed to investigate the involvement of annexin-1 in the healing of ulcers in the mouse stomach, integrating pharmacological observations with analyses in genetically modified mice.

GASTRIC ULCER HEALING IS A highly regulated process that involves inflammation, cell proliferation, formation of granulation tissue, and angiogenesis. For decades, agents that suppress acid secretion (H2 receptor antagonists and proton-pump inhibitors) have been widely used for promoting ulcer healing. However, there remains strong interest in better understanding the mechanisms through which ulcers heal and the possibility that both the speed and quality of healing may be pharmacologically enhanced (32).

Glucocorticoids are widely used, powerful anti-inflammatory agents, but they can significantly delay the healing of wounds (8, 21). Several human and animal studies have demonstrated that glucocorticoids depress angiogenesis and inhibit epithelial cell proliferation, which results in delayed gastric ulcer healing and an increase in the risk of ulcer perforation (5, 7, 16, 18). Annexin-1 is a 37-kDa member of the annexin family of proteins that bind to and activate “formyl-peptide” receptors (FPR) (3, 10, 19). The expression of this protein can be modulated by glucocorticoids and has been shown to account for a substantial part of the anti-inflammatory effects of glucocorticoids (13), largely through binding to and acting on a specific G protein-coupled receptor, termed formyl peptide receptor-like-1 (FPRL-1) (2, 10, 14). However, annexin-1 expression can also be regulated independent of glucocorticoids (26, 29). The ability of annexin-1 to interfere with lymphocyte and monocyte migration and to induce neutrophil apoptosis is considered important in the anti-inflammatory effects of this protein (12, 17, 20).

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influx and ischemia/reperfusion-induced cell adhesion and emigration (1, 11, 12). Boc2 (N-t-butoxycarbonyl-PLPLP) is an FPRL-1 antagonist (11, 24). Groups of at least five mice were treated twice daily intraperioneally with one of the following: vehicle (5% dimethyl sulfoxide), Ac2-26 (100 μg/kg), Boc2 (50 μg/kg), or dexamethasone (100 μg/kg). Treatment was initiated on day 3 following the induction of ulcers and continued for 2–4 days.

**Western blot analysis.** Western blot analysis of annexin-1 was performed by using gastric tissue from healthy mice or from mice at various time points following induction of ulcers. A sample of tissue that included the ulcer margin was snap frozen and stored at −80°C. The samples were homogenized in ice-cold lysis buffer composed of: 20 mM Tris·HCl, 0.1 mM PMSF, 5 μg/ml protease inhibitor cocktail (containing 4-[2-aminoethyl]benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotenin; Sigma-Aldrich, St. Louis, MO). Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). An aliquot of the lysate was placed into Laemmli’s sample buffer and boiled for 2 min. Equal amounts of protein (40 μg) were loaded onto a 10% gel, subjected to SDS-PAGE, and electrotransferred onto polyvinylide fluoride membranes (Hybond-P PVDF Membrane; Amersham Biosciences, Buckinghamshire, UK). Annexin-1 was detected by using a 1:1,000 dilution (24 h at 4°C) of a rabbit polyclonal antibody specific to annexin-1 (Zymed Laboratories, San Francisco, CA). The membrane was then incubated with HRP-conjugated anti-rabbit IgG (1:4,000) and detected by enhanced chemiluminescence on Hyperfilm ECL film (Amersham Biosciences). Band density was determined by using a calibrated imaging densitometer (GS-710, Bio-Rad) and Quantity One software (Bio-Rad). β-actin was used as an internal standard to ensure equal protein loading.

**Immunohistochemistry.** The streptavidin-peroxidase method was used for the immunolocalization of annexin-1. Briefly, following deparaffinization in xylene and rehydration in ethanol, the slides were washed three times in phosphate-buffered saline (PBS; pH 7.4) plus 0.5% Tween 20, and endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide (10 min). The tissue sections were then placed into a Copland jar filled with 10 mM citrate buffer (pH 6.0, 100°C) for 10 min to retrieve antigens. The sections were cooled for 30 min, washed three times in PBS + 0.5% Tween 20, and then incubated with normal goat serum for 20 min at room temperature. Tissue specimens were incubated overnight at 4°C in 1:100 anti-annexin-1 polyclonal antibody (Zymed Laboratories), followed by the addition of a biotinylated secondary antibody and streptavidin-HRP (Zymed Laboratories). 3, 3′-diaminobenzidine was used as a chromogen and hematoxylin for counterstaining. As a negative control, the same procedure was repeated except that the primary antibody was replaced by PBS + 0.5% Tween 20.

**Healing of indomethacin-induced gastric damage.** Experiments were first performed to determine whether annexin-1-deficient mice (generated on a C57BL6 background) (13, 23) exhibited different susceptibility to indomethacin-induced gastric damage than wild-type mice. Mice were fasted overnight, and then either vehicle or indomethacin (1, 3, 10 mg/kg; Sigma-Aldrich) was administered by oral gavage. Five hours later, the mice were euthanized and their stomachs removed and opened along the greater curvature. The length of hemorrhagic lesions was measured (in mm), and a gastric damage score was calculated as a summation of the lengths of all lesions in a stomach. The scoring of damage was performed by an individual blinded to the treatment each mouse received.

To determine whether annexin-1-deficient mice exhibited an impairment of healing of indomethacin-induced gastric damage, groups of annexin-1-deficient and wild-type mice (C57BL6; n = 10) were treated orally with indomethacin (10 mg/kg), and the severity of gastric damage was assessed in subgroups (n = 5) of mice 5 or 24 h later.

**Statistical analysis.** All values are expressed as means ± SE. Statistical analysis was performed by using one-way ANOVA followed by a Tukey’s post hoc test or Student’s t-test, where appropriate. An associated probability of less than 5% was considered as significant.

**RESULTS**

**Annexin-1 expression in healthy and ulcerated gastric tissue.** Application of acetic acid to the serosal surface of the mouse stomach resulted in the formation of discrete gastric ulcers that healed significantly during the 3–10-day period after their induction (Fig. 1A). Western blot analysis showed that the full-length annexin-1 protein (37 kDa), but not the 33-kDa cleavage product, was constitutively expressed in the normal mouse stomach (Fig. 1B). Following induction of an ulcer, there was a significant increase in expression of annexin-1 (37 kDa). There was also a significant increase in the expression of

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Fig. 1. Time course of gastric ulcer healing in mice (A) and changes in annexin-1 expression (B). Expression of annexin-1 in tissue from the margin of gastric ulcers was significantly increased compared with that in normal gastric tissue. As the antibody used recognizes cleaved and uncleaved annexin-1, the blot shows the expression of both the 37-kDa annexin-1 protein and the 33-kDa cleavage product. The histogram shows results of densitometric analysis of the expression of the full 37-kDa annexin-1 protein (data on the expression of the cleavage product are provided in RESULTS). *P < 0.05, **P < 0.01 compared with the control group (B). β-actin was used as an internal standard to ensure equal protein loading.
the 33-kDa cleavage product (indicative of ongoing inflammation) (31). Thus the expression (in arbitrary densitometry units) was $9.4 \pm 1.3$ in healthy controls vs., at days 3, 5, 7, and 10 postulcer, respectively, $51.4 \pm 2.9$, $70.2 \pm 6.4$, $79.6 \pm 4.9$, and $112.7 \pm 5.6$ (all $P < 0.05$ vs. healthy controls).

Localization of annexin-1 expression in healthy and ulcerated gastric tissue. Consistent with the Western blot analyses, the immunohistological data indicate that basal levels of annexin-1 protein are low in the gastric tissue from healthy mice (Fig. 2A). However, in the mice in which ulcers had been induced, there was strong staining for annexin-1, both in the ulcer bed and in the tissue surrounding the ulcer (Fig. 2, B and C). Annexin-1 staining was most evident in the cytoplasm of elongated fibroblast-like cells that were localized to the proliferating granulation tissue beneath the ulcer (Fig. 2C). Other cells that stained positive for annexin-1 likely include those that are morphologically compatible with monocytes, granulocytes, and smooth muscle cells. Parietal cells, chief cells, and mucous neck cells did not exhibit positive staining for annexin-1. Thus annexin-1 expression was most prominent in the regions where remodeling of gastric tissue was occurring (i.e., gastric gland and rugae reformation; Fig. 2D). It is important to note that the antibody used for immunohistochemical localization of annexin-1 recognizes both cleaved and uncleaved proteins.

Pharmacological modulation of ulcer healing. Twice-daily treatment with an annexin-1 mimetic (Ac2-26) for 2 days did not significantly affect the extent of ulcer healing compared with that observed in vehicle-treated controls (Fig. 3A). However, Ac2-26 treatment for 4 days resulted in a significant enhancement in ulcer healing (Fig. 3B). The reasons for this apparent delay in the onset of effect of Ac2-26 are not clear. In contrast, twice-daily treatment for 2 days with dexamethasone or with an FPRL-1 antagonist, Boc2, resulted in significant impairment of ulcer healing (Fig. 3A). This was a transient effect, however, since, after 4 days of treatment with dexamethasone or Boc2, the extent of ulcer healing was not significantly different from that observed in the vehicle-treated group. The beneficial effects on ulcer healing of 4 days of treatment with Ac2-26 was not observed if the mice were cotreated with Boc2 (mean ulcer area of $8.4 \pm 0.4 \text{ mm}^2$ vs. $13.9 \pm 0.9 \text{ mm}^2$, respectively; $n = 5$ per group).

Healing of indomethacin-induced gastric damage. Annexin-1-deficient and wild-type mice exhibited similar susceptibility to indomethacin-induced gastric damage (Fig. 4A). In normal mice, lesions of this type typically take 48–72 h to heal completely (J. L. Wallace, unpublished observations). When healing of indomethacin-induced erosions was examined in annexin-1-deficient mice, it was found to differ substantially from that in wild-type controls. Thus in wild-type mice, the

![Fig. 2. Immunohistochemical localization of annexin-1 in normal and ulcerated gastric tissue. Very little annexin-1 immunoreactivity was detected in normal tissue (A). The samples on the right side of the figure represent tissues that received the annexin-1 antibody, whereas those to the left are a sequential section that received no primary antibody (negative control). Annexin-1 expression was increased at sites near (B) and in the ulcer bed (C) during the healing of ulcers. Note the reepithelialization of the ulcer during the early phase of healing (C). The increase in annexin-1 staining corresponded with the remodeling of gastric tissue (D). The white bar in A represents 100 $\mu$m.]
Severity of mucosal injury decreased during the period from 5 h to 24 h after indomethacin administration (Fig. 4B). In annexin-1-deficient mice, the severity of gastric damage increased during the same period.

**DISCUSSION**

Annexin-1 has well-characterized anti-inflammatory effects and has been shown to contribute to gastric mucosal defense (34). The present study was performed to examine the potential role of annexin-1 in the healing of ulcerated gastric mucosa. Annexin-1 is expressed in the healthy gastric mucosa, and it is markedly upregulated following induction of an ulcer. Treatment with an annexin-1 mimetic improved gastric ulcer healing. Over the first 2 days of the healing process, but not thereafter, treatment with an FPRL-1 antagonist impaired ulcer healing. Although annexin-1-deficient mice did not exhibit any difference from wild-type mice in terms of susceptibility to indomethacin-induced gastric damage, the healing of those lesions was impaired in the annexin-1-deficient mice. These data are therefore consistent with the hypothesis that annexin-1 is an important contributor to the repair of damaged gastric mucosa.

Some of the anti-inflammatory effects of glucocorticoids, including inhibition of neutrophil adhesion in postcapillary venules, are mediated via the release of annexin-1 (20), and the majority of these effects are mediated via annexin-1 binding to and activating the FPRL-1 receptor (11, 24). This receptor is expressed on the surface of a variety of cells, including subepithelial myofibroblasts, smooth muscle cells, leukocytes, mast cells, and T cells (6). In the present study, the beneficial effects of annexin-1 also appear to be mediated via activation of the FPRL-1 receptor. Thus improvement of ulcer healing was observed in mice treated with an annexin-1 mimetic (Ac2-26), and this effect was reversed by concomitant administration of an FPRL-1 antagonist (Boc2). Interestingly, in addition to annexin-1, lipoxin A4 and aspirin-triggered lipoxin are also able to activate FPRL-1, and these eicosanoids also contribute to resolution of inflammation and to gastric mucosal resistance to injury (6, 33). The anti-inflammatory effects of annexin-1 are mediated through its NH2-terminal domain, since the proteolytic re-
mval of the NH₂ terminus results in its inactivation, whereas synthetic peptides corresponding to this region of annexin-1 exhibit full pharmacologic activity (6, 9, 12, 36). Thus we selected the synthetic peptide Ac2-26 as a pharmacological probe for our studies, since it elicits many of the effects of the full-length protein (26).

Concurrent with the increased expression of annexin-1 in the stomach of mice with ulcers, we observed an increase in expression of the 33-kDa cleavage product of annexin-1. Expression of this cleavage product was not observed in the healthy stomach, so it is likely that cleavage of annexin-1 occurred as a consequence of factors induced as part of the inflammatory and/or repair process. Annexin-1 cleavage has been observed in bronchoalveolar lavage fluids from patients with cystic fibrosis and in other lung diseases (25, 27, 30). In addition, intense expression and release of annexin-1 (mostly cleaved) has been observed in inflamed gut tissue (28). The increased expression of the annexin-1 cleavage product may be attributable to elevated levels of proteases, including elastase (22) and protease 3 (31). It is noteworthy that the latter is operative in the microenvironment of activated neutrophils and that gastric damage and repair is associated with marked neutrophil influx. The impact of NH₂-terminal cleavage in the biology of annexin-1 is unclear, i.e., it may be a catabolic phenomenon, or it might lead to the generation of anti-inflammatory mediators, including sequences similar to, or encompassing, peptide Ac2-26 (22). It is possible that the 33-kDa fragment may have similar anti-inflammatory properties as antiflammin-2, an annexin-1 core-derived peptide that is selective for the human FPRL-1 receptor (4, 15). The cleaved 33-kDa annexin-1 product would contain the antiflammin-2 region, and, if it produces biological actions, it will be providing a reiteration of this anti-inflammatory and proresolution circuit.

To some extent, the beneficial effects of annexin-1 on gastric ulcer healing that we have observed may be viewed as counterintuitive. As discussed above, many of the anti-inflammatory actions of glucocorticoids can be recapitulated with annexin-1 (13). However, glucocorticoids have long been known to interfere with the healing of gastric ulcers and to increase the risk of perforation (8, 21), possibly through their ability to depress angiogenesis and inhibit epithelial cell proliferation (5, 7, 16, 18). Thus actions of glucocorticoids independent of induction of annexin-1 expression and/or release may account for at least some of their untoward effects on gastric ulcer healing. Further studies are required to determine the annexin-1-independent actions of glucocorticoids and the extent to which such actions interfere with processes that contribute to ulcer healing.

In summary, we have demonstrated that annexin-1 expression is strongly induced in ulcerated gastric tissue (both in the ulcer bed and the ulcer margin). Moreover, administration of an annexin-1 mimetic improved ulcer healing, whereas an antagonist of the main receptor for annexin-1 impaired healing. We also report that the annexin-1-deficient mice show similar susceptibility to indomethacin-induced gastric damage. Significantly, however, the healing of that damage was impaired in the annexin-1-deficient mice compared with wild-type controls. Taken together, these data support the hypothesis that annexin-1 makes an important contribution to the healing of gastric mucosal damage. Gaining a better understanding of the mechanisms underlying these actions of annexin-1 could be beneficial in terms of the development of more effective therapies for the treatment of ulceration in the gastrointestinal tract and possibly wound healing in other tissues.

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