Hemostatic mechanisms, independent of platelet aggregation, arrest gastric mucosal bleeding

(mesenteric artery/prostacyclin/thromboxane synthase/heparin/ aspirin)

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ABSTRACT Platelet adhesion, aggregation, and subsequent plug formation play a major role in the control of cutaneous and vascular hemostasis. Little is known, however, about the hemostatic processes in gastric mucosal tissue. A method for evaluating bleeding from a standard incision in the gastric mucosa of the rat, rabbit, and dog has therefore been developed. By using pharmacological agents that interfere with platelet aggregation and blood coagulation, the mechanism of gastric hemostasis has been compared to that in the vasculature, using the rat mesenteric artery. Intravenous infusion of prostacyclin (0.5 µg·kg⁻¹·min⁻¹), which inhibits platelet aggregation directly, or administration of the thromboxane synthase inhibitor 1-benzylimidazole (50 mg·kg⁻¹) significantly prolonged bleeding in the mesenteric artery yet failed to alter gastric mucosal bleeding. In contrast, a low dose of heparin (100 units·kg⁻¹), which interferes with the clotting process, had no effect on mesenteric bleeding but substantially prolonged bleeding from the gastric mucosa. These findings suggest that, unlike in the skin or vasculature, platelet aggregation plays a minimal role in the initial hemostatic events in the gastric mucosa and that the arrest of gastric hemorrhage is brought about largely by processes primarily involving the coagulation system.

The adhesion and aggregation of platelets and their subsequent plug formation at the site of injury play a major role in the control of hemostasis in the skin and arterial vessel wall (1, 2). Although gastric biopsy is a routine procedure, and bleeding episodes from the upper gastrointestinal tract arising from peptic ulcer disease or following ingestion of drugs such as aspirin are common, little is known about the hemostatic processes in mucosal tissue. We have therefore developed a method for evaluating bleeding from a standard incision in the gastric mucosa of the rat, rabbit, and dog. Using this technique, the mechanism of hemostasis in the gastric mucosa has been compared to that in the rat mesenteric artery. Thus, the effects of parenteral administration of prostacyclin, which inhibits platelet aggregation directly (3), and of the thromboxane synthase inhibitor 1-benzylimidazole (BzI) (4, 5) on bleeding in the mesenteric artery and gastric mucosa have been studied. In addition, the effect of low doses of heparin, which interferes with the clotting process, has been investigated in order to evaluate the relative roles of platelet aggregation and blood coagulation in the hemostatic mechanisms of these two tissues.

METHODS

The method used to determine bleeding time in the gastric mucosa has been adapted from that used for such studies on the skin (6, 7). A standard incision was made in the gastric mucosa, whose surface was superfused with isotonic saline. To avoid local trauma to the mucosa, blotting techniques with filter paper, as used in the skin, were not used. The surgical techniques for exposing the gastric mucosa of the rat, rabbit, and dog were similar. Male Wistar rats (180-200 g) fasted 18–24 hr, were anesthetized with pentobarbitone (60 mg·kg⁻¹, i.p.) and the jugular vein was cannulated for intravenous drug or vehicle administration. The stomach was exposed by a midline laparotomy, opened along the greater curvature, and clamped in a perspex gastric chamber, ensuring that the vascular connections were intact (8). The perfusate (0.15 M NaCl at 37°C) was directed over the mucosal surface (3 ml·min⁻¹) and collected at 1-min intervals. Male rabbits (2.0–2.5 kg) were likewise anesthetized and, after tracheotomy, the jugular vein was cannulated. A midline laparotomy provided access to the stomach, which was opened along the anterior surface for a distance of 6 cm and the stomach was everted, thus providing a flap of gastric mucosa that could be placed in a chamber and the surface superfused with isotonic saline (3 ml·min⁻¹).

In further studies, anesthetized beagle dogs (10–12 kg) were intubated and ventilated and a segment of acid-secreting fundic mucosa was encased ex vivo in a plastic chamber, ensuring adequate vascular supply (5), and the mucosal surface was superfused at a rate of 6 ml·min⁻¹. The superfusion technique allowed the mucosal surface to be gently flushed, while the perfusate was not directed at the site of the lesion so as to reduce any potential disruption of the hemostatic process. Initial studies showed that the duration and extent of bleeding was not dependent on the rate of perfusion, and the rate chosen for each preparation was that which allowed for adequate washout of the chamber.

A 2-mm cut in the mucosa at a rugal fold with a number 12 surgical blade was used to initiate gastric mucosal bleeding in these preparations, with the hook-like shape of this blade allowing a standard incision to be made in the tissue. In an additional study, bleeding was initiated by a biopsy forceps, removing 7.7 ± 0.8 mg of tissue (n = 8). However, the blade incision was found to be more readily performed and reproducible and was therefore used in further studies.

For studies on bleeding in the rat mesenteric artery, a loop of ileum was placed over the edge of a chamber, which provided access to the vascular arcades of the mesentery (9–11). A binocular dissecting microscope was used to identify a branch of the mesenteric artery close to the ileum, and bleeding was produced by puncture with a 25-gauge needle (11). Isotonic saline (37°C) was superfused at 6 ml·min⁻¹ over the mesenteric vessel and collected at 30-sec intervals.

Bleeding was determined as the hemoglobin output (mg·min⁻¹) into the perfusate, using spectrophotometric

Abbreviations: BzI, 1-benzylimidazole; TXB₂, thromboxane B₂.

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techniques. Thus, the optical density ($A_{560m}$) of the hemoglobin in the perfusate was measured after lysis of the erythrocytes with 0.1 ml of Zaponin (Coulter) and calculated from a standard curve constructed with rat heparinized arterial blood diluted with saline. The bleeding time was taken as the time from the incision to the first collection period during which the hemoglobin output was <0.1 mg·min$^{-1}$, corrected for the chamber washout time.

Data are shown as means ± SEM of $n$ values, where the level of statistical significance was determined by Student's $t$ test, taking $P < 0.05$ as significant.

RESULTS

The hemoglobin output during the initial collection period was similar in all experimental groups and diminished rapidly from the time of the mucosal incision (Fig. 1). Direct observation of the cessation of bleeding via a binocular microscope gave values comparable with those determined by hemoglobin output. In studies in the rat, the bleeding time during saline superfusion of the gastric mucosal surface (3.4 ± 0.2 min; $n = 13$) was comparable to that during superfusion with acidified saline (pH 2) containing 0.15 M HCl (3.7 ± 0.16 min; $n = 10$), indicating that high concentrations of luminal acid do not affect gastric hemostasis. During saline superfusion, the initial hemoglobin output after incision was 5.1 ± 0.8 (n = 13), 1.1 ± 2.1 (n = 6), and 11.6 ± 3.6 mg·min$^{-1}$ (n = 4) for the rat, rabbit, and canine gastric mucosa, respectively, while the bleeding time was comparable in all three species (Table 1). The initial hemoglobin output after biopsy tissue removal from rat gastric mucosa during saline superfusion (3.6 ± 0.7 mg·min$^{-1}$; $n = 8$) was comparable to that during acid saline (pH 2) superfusion (3.2 ± 0.4 mg·min$^{-1}$; $n = 8$), confirming the findings with the incision; there was no significant difference in bleeding time after any of these procedures. All further studies were conducted by using saline superfusion and the blade incision.

The initial hemoglobin output following puncture of the rat mesenteric artery (12.0 ± 1.9 mg·min$^{-1}$; $n = 11$) diminished rapidly in the ensuing collection period and was comparable in all experimental groups studied, while the control bleeding time was similar to that in the gastric mucosa (Table 1).

**Effects of Inhibition of Platelet Aggregation.** Intravenous infusion of prostacyclin (0.5–5.0 μg·kg$^{-1}$·min$^{-1}$, EpoprostenoI; Wellcome), commenced 10 min prior to and maintained throughout the observation period in doses that have been demonstrated to inhibit near-maximally the ex vivo platelet aggregation in these species (12), failed to alter significantly the bleeding time in rat, rabbit, or dog gastric mucosa (Table 1). In contrast, intravenous infusion of prostacyclin (0.5 μg·kg$^{-1}$·min$^{-1}$) significantly ($P < 0.05$) prolonged the bleeding time following puncture of the rat mesenteric artery (Table 1).

**Effects of Thromboxane Synthase Inhibition.** Intravenous administration of Bzi as the fumarate salt (50 mg·kg$^{-1}$; Wellcome) 10 min prior to puncture significantly prolonged bleeding from the rat mesenteric artery (Table 1). In contrast, Bzi in this dose had no significant effect on the bleeding time in the gastric mucosa in any of the three species (Table 1). This dose of Bzi inhibited the formation of thromboxane B$_2$ (TXB$_2$) in clotting arterial blood from rat, rabbit, and dog by 90% ± 3%, 91% ± 1%, and 74% ± 9%, respectively (Table 2).

Local application of aspirin (20 mM) in 0.15 M HCl 10 min prior to incision of the rat gastric mucosa also failed to alter significantly the bleeding time (3.9 ± 0.45 min; $n = 6$). Furthermore, intravenous administration of indomethacin (10 mg·kg$^{-1}$) 10 min prior to incision likewise failed to alter the gastric bleeding time (3.8 ± 0.3 min; $n = 4$).

**Effects of Heparin.** Intravenous injection of heparin (100 units·kg$^{-1}$) significantly prolonged ($P < 0.001$) the bleeding from the gastric mucosa, which did not cease within the 15-min observation period (Fig. 1). In contrast to gastric mucosal bleeding, this dose of heparin did not alter significantly either the bleeding time in the rat mesenteric artery or the pattern of hemoglobin output (Table 1).

**Histological Appearance.** In a preliminary comparative histological study using five rats, the mesenteric artery was fixed at various time intervals after puncture, embedded in plastic, stained, and sectioned for evaluation by light microscopy (Fig. 2). The presence of platelet aggregates was apparent at the site of damage within 15–30 sec of puncture of the vessel wall, with extensive platelet plugging of the arterial lesion after 3–4 min, when bleeding had terminated. In sections of the gastric mucosa similarly processed from five rats, no such platelet aggregates could be observed at the site of incision after 1–2 min, and only small platelet clumps were seen in the fibrinous coagulated mass that plugged the mucosal wound after 4 min (Fig. 2).

**DISCUSSION**

Direct observation of hemostatic plug formation and the major characteristics of the hemostatic process were described over 100 years ago (14, 15). Many studies since that time have established that the primary arrest of bleeding from both the skin and small blood vessels is dependent on platelet adhesion to the cut surface and consequent aggregation, with later participation of the coagulation system and fibrin strand formation in stabilization of the plug (1, 7, 16, 17). In the present studies, we have investigated the mechanism that terminates gastric mucosal bleeding. Similar rates of hemoglobin output and time for cessation of gastric mucosal bleeding were observed whether bleeding was initiated by a linear incision with a surgical blade or by removal of a plug of mucosal tissue with a biopsy forceps. Comparative studies were performed on the rat mesenteric artery. This technique has been demonstrated to be a reliable model of the general hemostatic process that arrests bleeding from...
vascular and cutaneous sites following puncture or incision and is dependent on platelet adhesion and aggregation (9-11).

Prostacyclin inhibits the aggregation of platelets, both in vivo and in vitro, induced by all endogenous agents (5, 12) and therefore would be expected to delay the hemostatic processes dependent on platelet aggregation. Thus, in our studies, intravenous infusion of prostacyclin significantly prolonged the bleeding time following puncture of the rat mesenteric artery. In contrast, intravenous infusion of prostacyclin in doses that have been demonstrated to inhibit near-maximally the ex vivo platelet aggregation in these species (12) failed to alter significantly the bleeding time in either rat, rabbit, or dog gastric mucosa.

Thromboxane A2 from platelets has been implicated as an endogenous proaggregatory mediator. Inhibition of its synthesis can reduce platelet aggregation in vitro (18) and prolong cutaneous bleeding time in humans (19, 20). Intravenous administration of Bzi in a dose sufficient to cause near-maximal inhibition of TXB2 formation in clotting blood significantly prolonged the bleeding time in the rat mesenteric artery. In contrast, Bzi had no significant effect on the bleeding time in the gastric mucosa in any of the three species. Furthermore, intravenous administration of indomethacin, in a dose known to inhibit both platelet and mucosal cyclooxygenase (21), failed to alter the gastric bleeding time in these species. Thus, inhibition of endogenous thromboxane formation, either by a selective thromboxane synthase inhibitor or by cyclooxygenase inhibition, does not delay the gastric hemostatic process, while alterations in gastric mucosal prostacyclin formation after aspirin or indomethacin administration are also without effect.

It has been proposed that the gastric ulcerogenic actions of aspirin and similar compounds result from a synergistic interaction between the well-documented local irritant actions exerted directly on the mucosal cells and the inhibition of the gastric cyclooxygenase (22, 23). The latter action, by inhibiting the synthesis of endogenous protective prostanooids, makes the mucosa considerably more susceptible to damage (21, 22). Since neither direct inhibition of platelet aggregation nor topical application of aspirin altered gastric bleeding time in the current study, we conclude that interference with platelet aggregation plays little part in the bleeding associated with the gastric irritancy that follows administration of nonsteroid anti-inflammatory agents. Our results now explain the findings of an earlier clinical study in which acute or chronic aspirin administration failed to enhance significantly mucosal bleeding from gastric biopsy sites in human volunteers in doses sufficient to prolong the skin bleeding time (24). These observations support our concept that a hemostatic mechanism, largely independent of platelet aggregation, exists in the human gastric mucosa, as found in the gastric mucosa of the rat, dog, and rabbit.

Intravenous injection of heparin (100 units/kg) substantially prolonged the bleeding time from the rat, rabbit, and dog gastric mucosa, which did not cease within the 15-min observation period. The initial decline in hemoglobin output from the gastric mucosa, which was unaffected by any of the agents including heparin, may reflect local changes in microcirculatory tone following incision. In contrast to gastric mucosal bleeding, this dose of heparin failed to alter significantly the bleeding time in the rat punctured mesenteric artery. In earlier studies on the rat mesenteric artery, higher doses of heparin likewise did not consistently prolong primary bleeding time (9). Thus, interference of the clotting mechanisms with this dose of heparin selectively prolongs gastric mucosal bleeding without affecting mesenteric bleeding. In this context, it is pertinent that both the hemorrhagic complications following anticoagulant treatment, extensive bleeding from undiagnosed peptic ulceration can account for a significant proportion of the deaths from such therapy (25).

Skin bleeding time following a standard incision is used as a reliable diagnostic indicator of the hemostatic capabilities of platelets in humans (1, 6, 7). Thus, skin bleeding time can be prolonged by inhibitors of platelet aggregation such as aspirin (6, 7, 19, 24, 26), but not by inhibition of the coagulation cascade by heparin (16). Furthermore, hemophiliacs who have a normal platelet function in gastric biopsy sites exhibit a normal or near-normal primary skin bleeding time (16, 27). The present studies with inhibitors of platelet aggregation and with heparin on mesenteric arterial bleeding in the rat are in accord with these hemostatic schemes. In marked contrast, the primary termination of gastric mucosal bleeding appears to be less dependent on platelet aggregation than on the coagulation system, which may account for the frequent clinical observation of bleeding from the stomach or duodenum in hemophiliac patients (28).

The histological study of the mucosal incision at a time when bleeding had ceased showed a fibrinous coagulated mass that plugged the wound with minimal involvement of platelet aggregates. This contrasted with the extensive platelet plugging of the mesenteric arterial lesion, as also observed

Table 1. Bleeding times in the rat, rabbit, and dog

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Prostacyclin</th>
<th>Bzi</th>
<th>Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>3.4 ± 0.4 (4)</td>
<td>3.7 ± 0.5 (4)</td>
<td>3.4 ± 0.5 (4)</td>
<td>&gt;10*** (4)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3.6 ± 0.2 (6)</td>
<td>3.6 ± 0.2 (6)</td>
<td>3.6 ± 0.2 (6)</td>
<td>&gt;10*** (3)</td>
</tr>
<tr>
<td>Rat</td>
<td>3.4 ± 0.2 (13)</td>
<td>4.0 ± 0.6 (13)</td>
<td>4.1 ± 0.4 (8)</td>
<td>&gt;15*** (6)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>3.0 ± 0.2 (7)</td>
<td>6.4 ± 0.9* (6)</td>
<td>6.1 ± 0.7* (6)</td>
<td>3.3 ± 0.6 (6)</td>
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Bleeding time (in minutes) in the gastric mucosa after a standard 2-mm incision and in the rat mesenteric artery after puncture with a 25-gauge needle was determined by hemoglobin output. The actions of intravenously administered prostacyclin (0.5 μg·kg⁻¹·min⁻¹ in rat and dog and 0.8 μg·kg⁻¹·min⁻¹ in rabbit), Bzi (50 mg·kg⁻¹), and heparin (100 units·kg⁻¹) on the bleeding time are shown. The results (which have been corrected for the chamber washout time as determined by addition of heparinized rat blood) are mean ± SEM of (n) experiments, where difference from control is shown as *P < 0.05 and ***P < 0.001.

Table 2. Inhibition of serum thromboxane formation by Bzi

<table>
<thead>
<tr>
<th>TXB2, ng·ml⁻¹</th>
<th>Control</th>
<th>Bzi</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>292 ± 13</td>
<td>29 ± 7***</td>
<td>5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>420 ± 47</td>
<td>37 ± 4***</td>
<td>4</td>
</tr>
<tr>
<td>Dog</td>
<td>582 ± 83</td>
<td>154 ± 50**</td>
<td>3</td>
</tr>
</tbody>
</table>

Bzi (50 mg·kg⁻¹, i.v.)-induced inhibition of serum levels of TXB2 from rat, rabbit, and dog. Arterial whole blood (1 ml) was collected in glass tubes 10 min after Bzi administration and allowed to clot for 45 min at 37°C. Indomethacin (10 μg) was added to each tube and centrifuged for 10 min (1000 × g at 4°C), and TXB2 was determined by radioimmunoassay (13). Results are shown as mean ± SEM of (n) values where **P < 0.02 and ***P < 0.001.
FIG. 2. Histological appearance of the rat gastric mucosa following incision and of the mesenteric artery after puncture. The gastric chamber was flooded with a buffered fixative solution (4% formaldehyde/1% glutaraldehyde in phosphate buffer, pH 7.2) 4 min after making the incision, and the segment of tissue was removed and stored in the fixative solution. Likewise, 15 sec after puncture, the mesenteric artery was immersed in the fixative solution, and the tissue segment was removed and stored. Using routine procedures, the tissues were embedded in Spurr epoxy resin, and 1-μm sections were prepared and stained with methylene blue azure II basic fuchsin. (Upper) Rat mesenteric artery with extensive platelet aggregates (P) surrounding the area of puncture (I), with the early platelet plug almost occluding the wound. Erythrocytes (RBC) can be seen outside the vessel wall (V) and within the vessel lumen (L). (×340.) (Lower) Incision (I) in the gastric mucosa (M), where S are surface epithelial cells, with extensive bleeding into the submucosal tissue. Although at this time bleeding had ceased, the wound contained only a few platelet aggregates (P) within the mass of coagulated erythrocytes (RBC) and fibrin, with a thin layer of platelet aggregates at the outer perimeter of the plug. (×380.)

in histological studies on both vascular and cutaneous lesions (1, 7, 9, 16, 17). These observations give support to the conclusions drawn from the pharmacological studies indicating the importance of the coagulation system in the primary control of gastric hemorrhage.

In the vessel wall and in the skin, formation of the
hemostatic plug, which is dependent on platelet aggregation, occurs away from the predominant site of prostacyclin production, the vascular endothelium (3). However, in the gastric mucosa, a tissue with a high capacity for prostacyclin biosynthesis (21), nature appears to have developed an alternative mechanism of hemostasis that does not rely on platelet aggregation. Although two distinct hemostatic mechanisms operate in the stomach and arterial vascularity, the duration of bleeding at both sites was similar, an interesting example of alternative regulatory processes mediating a physiological response. Our findings thus have implications for the therapeutic management of gastric mucosal hemorrhage and for understanding the gastrointestinal side effects of antiplatelet and anticoagulant therapy. It remains to be investigated whether a mechanism similar to that of the gastric mucosa operates or contributes in other mucosal surfaces such as the uterus, where the process of primary hemostasis is not clearly understood (29, 30).

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