ORIGINAL ARTICLE

Arteriovenous shunting blood flow is intravitally observed in the stomach after thermal injury in rats

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Abstract. Microcirculatory disturbance of the gastric wall is a crucial factor in the development of gastric mucosal lesions induced by *Helicobacter pylori*, anti-inflammatory drugs and stress. Opening of the arteriovenous shunting channel after thermal injury is one of the possible mechanisms to reduce mucosal blood flow. However, no *in vivo* observation of arteriovenous shunting blood flow in the stomach has been reported. To assess gastric microcirculatory disturbance, especially arteriovenous shunting blood flow after thermal injury by *in vivo* microsciopy, male Wistar rats were anesthetized and thermal injury was inflicted on the back skin. Gastric microvascular images were observed by *in vivo* microscopy. Rolling of leukocytes labelled with carboxyfluorescein diacetate, succinimidyl ester were counted and blood flow dynamics were observed by flow of a micro dye, monastral blue B (MBB). The endothelial damage was assessed by deposits of MBB 5 min after the administration. Arteriovenous shunting blood flow is difficult to detect by normal methods, but it could be observed by flow of MBB after thermal injury. Statistical analysis showed a significant difference in the ratio of arteriovenous shunting blood flow detection between the control (no injury) (0%; n = 15) and thermal injury (5 hrs after thermal injury) (28.6%; n = 14) groups. In the thermal injury group, the percentage of rolling leukocytes and the area of monastral blue B deposits increased, and the venular walls tended to be irregular. The total length of erosion increased time-dependently after thermal injury, and the length at 5 hrs was approximately 2 times larger than that at 2 hrs. Arteriovenous shunting blood flow is intravitally observed after thermal injury. A-V shunting blood flow can be a cause of mucosal hypo-perfusion. It is suggested that the microcirculatory disturbance seen 5 hrs after thermal injury is contributed to the final step of erosion formation. (Keio J Med 51 (4): 193–200, December 2002)

Key words: burn, endothelial damage, rolling of leukocytes, arteriovenous shunt, stress

Introduction

Microcirculatory disturbance of the gastric wall is a crucial factor in the development of gastric mucosal lesions induced by *Helicobacter pylori*,[1–3] anti-inflammatory drugs[4,5] and stress.[6,7] After thermal injury to the dorsal skin in rats, macroscopic hemorrhagic erosion developed in 14.3% at 15 min, 42.9% at 2 hrs, 100% at 5 hrs, and 85.7% at 12 hrs.[8] Macroscopic hemorrhagic erosion is formed within 5 hours after thermal injury. Under the stereoscopic microscope, superficial gastric erosion could be observed in all rats studied at 15 min. Gastric mucosal blood flow decreased at 15 minutes, partially improved at 2 hrs, and decreased again at 5 hrs after thermal injury.[9] Since the blood flow was depressed especially at 15 minutes and 5 hours, we have investigated mechanisms of the decrease in gastric mucosal blood flow at 15 minutes and 5 hours. Silicon rubber casts of the vasculature revealed contraction of arterioles 15 min after thermal injury to the dorsal skin in the rat model.[10] We also observed the gastric microvessels by *in vivo* microscopy which was first described by Guth and Rosenberg.[11,12] *In vivo* microscopic observation of gastric micro-

circulation showed also the contraction of arterioles 15 min after thermal injury.[13] The contraction is responsible for the decrease in gastric mucosal blood flow 15 min after thermal injury. Five hours after thermal injury, *in vivo* microscopy showed irregular constriction of the venules caused by suppressed production of nitric oxide.[14] Another interesting finding of the microcirculation 5 hrs after thermal injury is the opening of arteriovenous (A-V) shunting channels, as demonstrated by silicone rubber casts[10,15] and *in vivo* microscopy.[9] A-V shunting channels in the submucosa or base of the gastric mucosa are thought to divert blood flow from the mucosa and thereby contribute to mucosal lesion formation. No study, however, has demonstrated direct observation of A-V shunting blood flow, which is difficult to identify by ordinary *in vivo* observation.

Monastral blue B (MBB) is a stable pigment of negligible solubility in water which deposits in the vessels where permeability is increased.[16] MBB has previously been used to assess gastric mucosal microcirculatory disturbance[17] using permanent specimens. We applied MBB to *in vivo* observation of gastric microcirculation, with the idea that observation of both deposits and flow of MBB would allow assessment of endothelial damage and A-V shunting blood flow. One factor inducing endothelial damage and vascular hyperpermeability which may be assessed by MBB is leukocyte-endothelial interaction including the rolling of leukocytes.[18] We have previously observed the behavior of leukocytes 5 hrs after thermal injury using acridine orange.[9] However, fluorescent illumination of leukocytes after administration of acridine orange was rapidly lost and rolling of leukocytes could be counted for only 30 sec. In the present study, we attempted to observe the rolling of leukocytes labelled with 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFDASE). CFDASE is a nonfluorescent precursor that diffuses into cells and forms a stable fluorochrome, carboxyfluorescein succinimidyl ester (CFSE), after being catalyzed by an esterase which occurs predominantly in leukocytes and platelets.[19] CFSE fluorescence provides for a extended duration of *in vivo* observation.

The present study attempted to assess gastric microcirculatory disturbance 5 hrs after thermal injury using MBB and CFDASE. We were particularly interested in investigating whether it is possible to observe A-V shunting blood flow directly using *in vivo* microscopy.

Materials and Methods

Animals and procedures

Male Wistar rats weighing 220–270 g were fasted for 24 hours with free access to water before the experiments. They were anesthetized with diethyl ether, and a 30% full skin-thickness dorsal scald burn was caused by placing them in scalding water for 15 seconds as described previously.[20] The animals showed no reaction to pain. The animal experimentation guidelines of the Keio University School of Medicine were followed. The experiment was performed on two groups: control group (n = 15) (without thermal injury) and thermal injury group (n = 14) (5 hrs after thermal injury).

Gastric microvascular images

Microvascular images were observed with an intravital microscope as described previously.[21,22] Animals in both groups were anesthetized with sodium pentbarbital (20 mg/kg im), and laparotomy was performed just before the observation. A portion (about 2 mm in diameter) of the serosa and muscle layer of the gastric wall was resected gently, the stomach was mounted on a plastic stage, and a light rod was inserted through an incision in the forestomach. Microcirculatory images were observed with 10× objective, captured with a charge-coupled video camera system (CC-980; Flovel, Tokyo, Japan) and recorded on S-VHS videotapes.

Rolling leukocytes

Before thermal injury was inflicted, 0.1 ml of 15.6 mM/L 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFDASE) (Molecular Probes, Eugene, OR, USA) was administered intravenously to label leukocytes. Illuminated cells were monitored by fluorescence microscopy using a silicon intensifier target image tube camera (Hamamatsu Photonics, Shizuoka, Japan) according to a method described previously.[23] The leukocytes which stick to the same place for more than 30 sec were defined as sticking. Leukocytes rolling along the wall of venules in the basal region of the gastric mucosa were observed on videotaped images. Leukocytes passing through the confluence of a prevenule and a venule were counted for 3 min in the field observed with a 10× objective and the percentage of rolling leukocytes was calculated as the number of the rolling leukocytes/the number of leukocytes passing through the confluence of prevenules and venules ×100 (%), as described previously.[13] Since the number of circulating leukocytes can change, we decided to calculate the percentage. Leukocytes were counted at three different confluences in each rat and the average values were compared.

Vascular labeling with monastral blue B

Three percent suspensions of MBB (Sigma Chemical Co., St Louis, MO, USA) were administered intravenously (0.1 ml/100 g body weight) as described pre-

viously.[16] Deposits of MBB in venules were then observed intravitally with a CCD camera 5 min after administration. The percentage of the area of MBB deposits in venules in two fields (6× objective) was calculated using NIH Image software version 1.56 (Aladdin systems Co, Watsonville, USA) on a Macintosh computer.

Intravital observation of arteriovenous shunting of blood flow

MBB deposits were the results of the endothelial damage. We carefully observed the flow of MBB for the detection of the A-V shunting blood flow for 5 min after administration. It was reported that the mucosal venules connected with the mucosal capillary network only at, or very close to, the luminal surface and passed through the mucosa without receiving further tributaries, i.e. all mucosal capillary blood must drain via vessels close to the stomach lumen.[24] Therefore, any blood flow draining at the base of the mucosa without flow into the mucosal layer can be recognized as A-V shunting blood flow. A-V shunting blood flow can be identified if a venule accepts blood flow from an arteriole at the base of the gastric mucosa without flow into the mucoosal layer. A-V shunting of blood flow was recognized only in cases in which both the outflow of MBB from the arteriole and the inflow of the same MBB grain into the venule were observed.

Assessment of the mucosal lesions

Animals were killed at 15 min (n = 8), 2 hrs (n = 8) and 5 hrs (n = 12), and the length of erosions was measured with stereoscopic microscopy and the total length of all erosions per rat (TLE) was calculated.
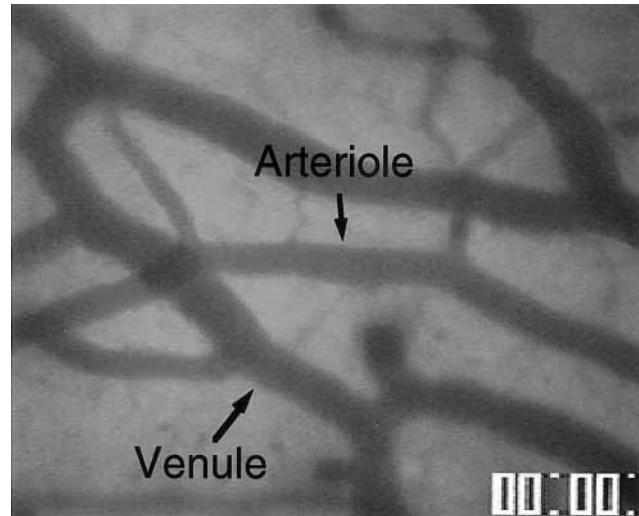
Statistical analysis

Data were presented as means + S.D. Statistical significance of the differences between two groups was determined by the Mann-Whitney test and Fisher's exact test.
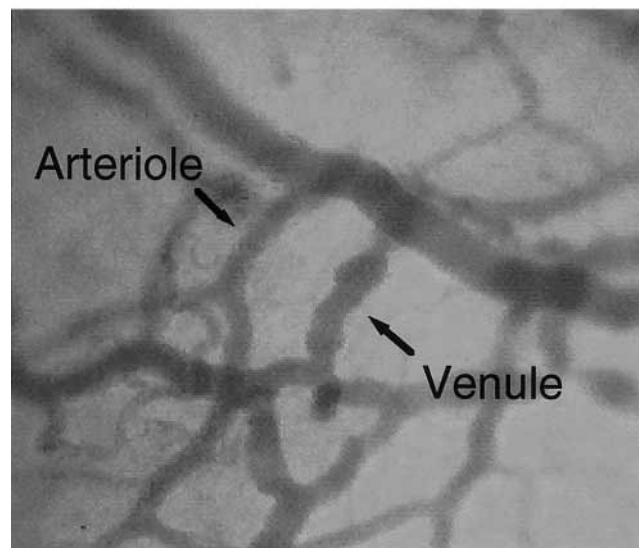
Results

Gastric microvascular images

The walls of arterioles and venules in the control group were basically smooth and there was no evident contraction during the experiment up to 30 min (Fig. 1A). In the thermal injury group, irregularity of the venular walls and abnormal venular constrictions were always observed, but arteriolar constriction was rarely observed (Fig. 1B).

Fig. 1 Gastric microvascular images. 1A: The walls of control arterioles and venules. 1B: Irregularity of the venular walls and abnormal venules in the thermal injury group.

Rolling leukocytes

Fluorescence of leukocytes was sufficiently stable for long observation and counting the number of leukocytes for 3 min was reproducible. The percentage of rolling leukocytes which passed the confluence of prevenules and venules was $1.9 + 3.0\%$ in the control group and $20.8 + 7.1\%$ in the thermal injury group, with this difference significant ($p < 0.01$) (Fig. 2).
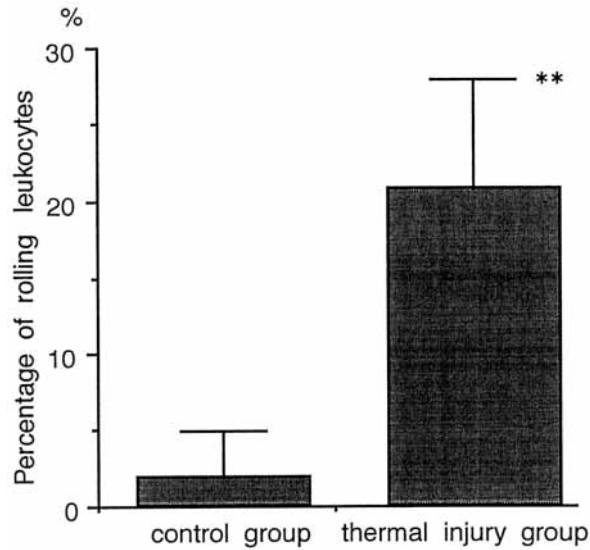
Fig. 2 Percentage of rolling leukocytes. Leukocytes passing though the confluence of a prevenule and venule were counted for 3 min and the percentage of rolling leukocytes was calculated. Values significantly increased 5 hrs after thermal injury (** $P < 0.01$).
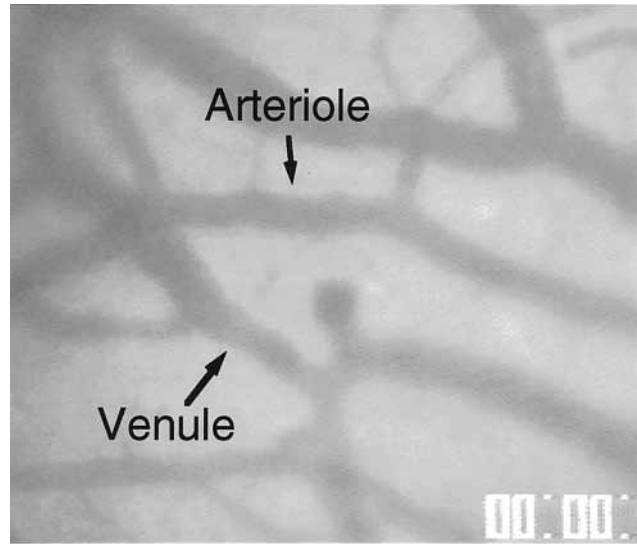
Vascular labeling with monastral blue B

Few venular deposits of MBB were seen in the control group but were evident in the thermal injury group (Fig. 3), with the percentage area of MBB deposits in venules being $0.7 + 1.3\%$ in the control group and $13.4 + 12.7\%$ in the thermal injury group ($p < 0.01$) (Fig. 4).

Intravital observation of arteriovenous shunting

Although it was difficult to demonstrate the continuity of A-V shunting channels from arterioles to venules by two dimensional observation, A-V shunting was identified using MBB. Both the outflow of MBB from an arteriole and the inflow of the same grain of MBB into a venule were observed in the thermal injury group (Fig. 5). While A-V shunting of blood flow was not observed in any animal in the control group ($n = 15$), arteriovenous shunting of blood flow was detected in 4 of 14 rats in the thermal injury group, the difference being statistically significant ($p < 0.05$) (Table 1).

Gastric mucosal erosion

The total length of erosions was 1.80 ± 1.42 mm at 15 min, 3.35 ± 2.14 mm at 2 hrs and 6.33 ± 5.04 mm at 5 hrs after thermal injury. It increased time-dependently after thermal injury (Fig. 6), and the length at 5 hrs was approximately 2 times larger than that at 2 hrs.
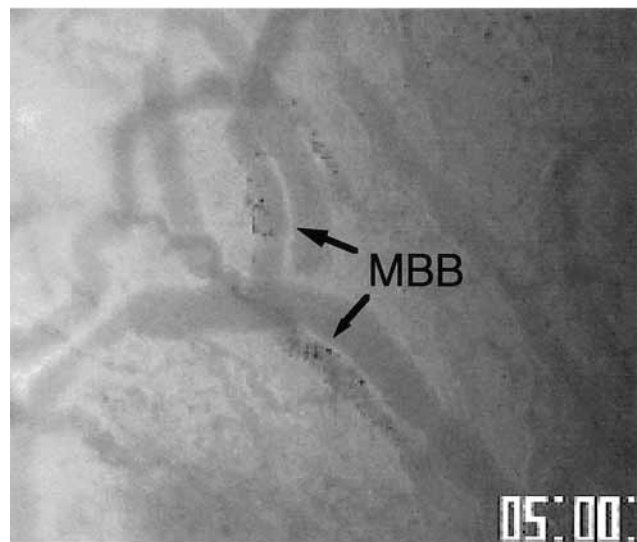
Fig. 3 Vascular labelling with Monastral Blue B (MBB). 5 min after administration of 3% suspension of MBB (0.1 ml/100 g body weight). 3A: Few venular deposits of MBB are seen in the control group. 3B: Venular deposits of MBB are clearly evident in the thermal injury group.

Discussion

Although gastric A-V shunting channels after thermal injury have been demonstrated using a silicone rubber cast of the vasculature,[15] their presence has been regarded as controversial. A-V shunting blood flow is difficult to identify by ordinary *in vivo* observation. After the application of MBB, however, it was
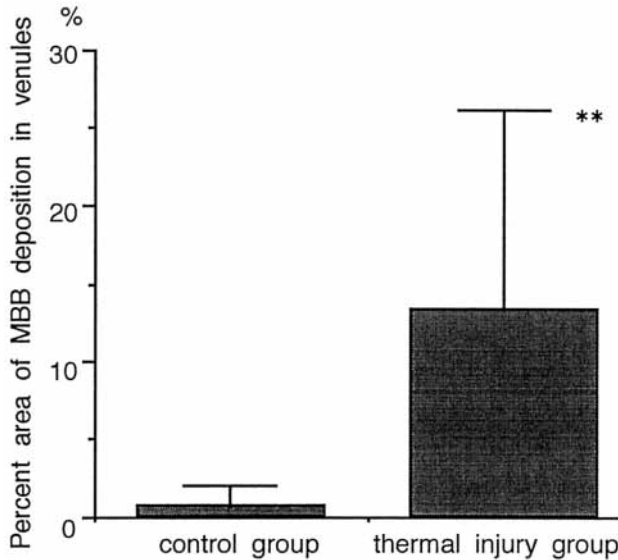
Fig. 4 Percent area of MBB deposits in venules. Percent area of MBB deposits in venules in two fields (60×) was calculated. The percentage of MBB deposits in the thermal injury group was significantly higher than that of the control group (p < 0.01).

possible to observe A-V shunting blood flow directly in the present study.

Because serosal resection could not be larger than 2 or 3 mm in diameter in order to avoid bleeding, we observed only a limited area of the stomach. Notwithstanding this methodological limitation, statistical analysis showed a significant difference in the detection of A-V shunting blood flow. The present results support those of studies that used silicone rubber casts of the vasculature.[10,15] A-V shunting were observed in both methods after thermal injury. A-V shunting blood flow in the basal region of the gastric mucosa or submucosal layer would steal blood from the mucosa, supported by the observation that gastric mucosal blood flow was decreased 5 hrs after thermal injury.[25] Macroscopic hemorrhagic erosion was formed within 5 hours after thermal injury,[8] and the total length of erosions measured using stereoscopic microscope increased time-dependently after thermal injury. Both the incidence[8] of macroscopic hemorrhagic and the total length of erosion at 5 hrs were 2 times larger than that at 2 hrs. The decrease in the gastric mucosal blood flow 5 hrs after thermal injury plays a role in the final step of erosion formation. A-V shunting blood flow is the totally abnormal blood flow which does not reach most of mucosal cells and cannot contribute to the mucosal defensive mechanism.

Our result showed that rolling of leukocytes in venules was also increased 5 hrs after thermal injury. Mulligan et al.[26] reported that both vascular injury and remote lung injury after thermal injury are neutrophil-

dependent and require the participation of E-selectin and L-selectin. These adhesion molecules are probably responsible for the increase in rolling leukocytes observed in our study. Administration of P-selectin-neutralizing monoclonal antibody decreases plasma free amino-nitrogen concentration in a model of splanchnic ischemia-reperfusion, suggesting the presence of leukocyte-dependent tissue injury.[27] The addition of leukocytes stimulated by phorbol myristate acetate to an endothelial cell monolayer causes a significant increase in the intracellular peroxide level in the endothelial cells after 15 min and severe endothelial cell injury after 5 hrs, suggesting the presence of leukocyte-dependent endothelial damage.[28] We previously reported an increase in active oxygen species generated by leukocytes obtained from the gastric vein and inferior vena cava 5 hrs after thermal injury.[8] These previous studies indicated that leukocyte-dependent endothelial damage probably exists 5 hrs after thermal injury and an important factor in formation of gastric mucosal lesion. Therefore we attempted to show in vivo the existence of endothelial damage in the present study.

It has previously been reported that MBB is able to label a leaky vessel if its endothelial barrier is interrupted without being totally disrupted.[16] Little venular deposition of MBB was seen in the control group, but was evident in the thermal injury group. This finding indicates that the endothelial barrier of venules may be interrupted after thermal injury due to endothelial cell damage. In our previous reports, percentages of the rolling leukocytes observed 15 min after thermal injury were approximately 27–29%.[7,13,29] The values were a little higher than that observed 5 hrs (20.8%) after thermal injury in the present study. However, the percentage area of MBB deposits in venules observed 15 min after thermal injury was approximately 6%.[13,29] The values were lower than that observed 5 hrs (13.4%) after thermal injury in the present study. Rolling of leukocytes is considered to be a first step of leukocyte-endothelial interactions and subsequently followed by aggravation of the endothelial damage.

We also observed irregularity of venule walls at 5 hrs after thermal injury. A similar condition was observed when preparation for intravital microscopy was not successful with bleeding from the serosal incision. However, irregularity was always observed at 5 hrs after injury with sufficient preparation without bleeding. Our previous report showed no evident change in venular walls at 15 min after thermal injury.[13] Therefore, this irregularity represents a pathological reaction induced by thermal injury. We thought that dehydration might be a cause of this irregularity, and performed the same experiment with water resuscitation. Because this venular irregularity was also observed in water resuscitated rats,[30] dehydration is not a major cause of the irregu-
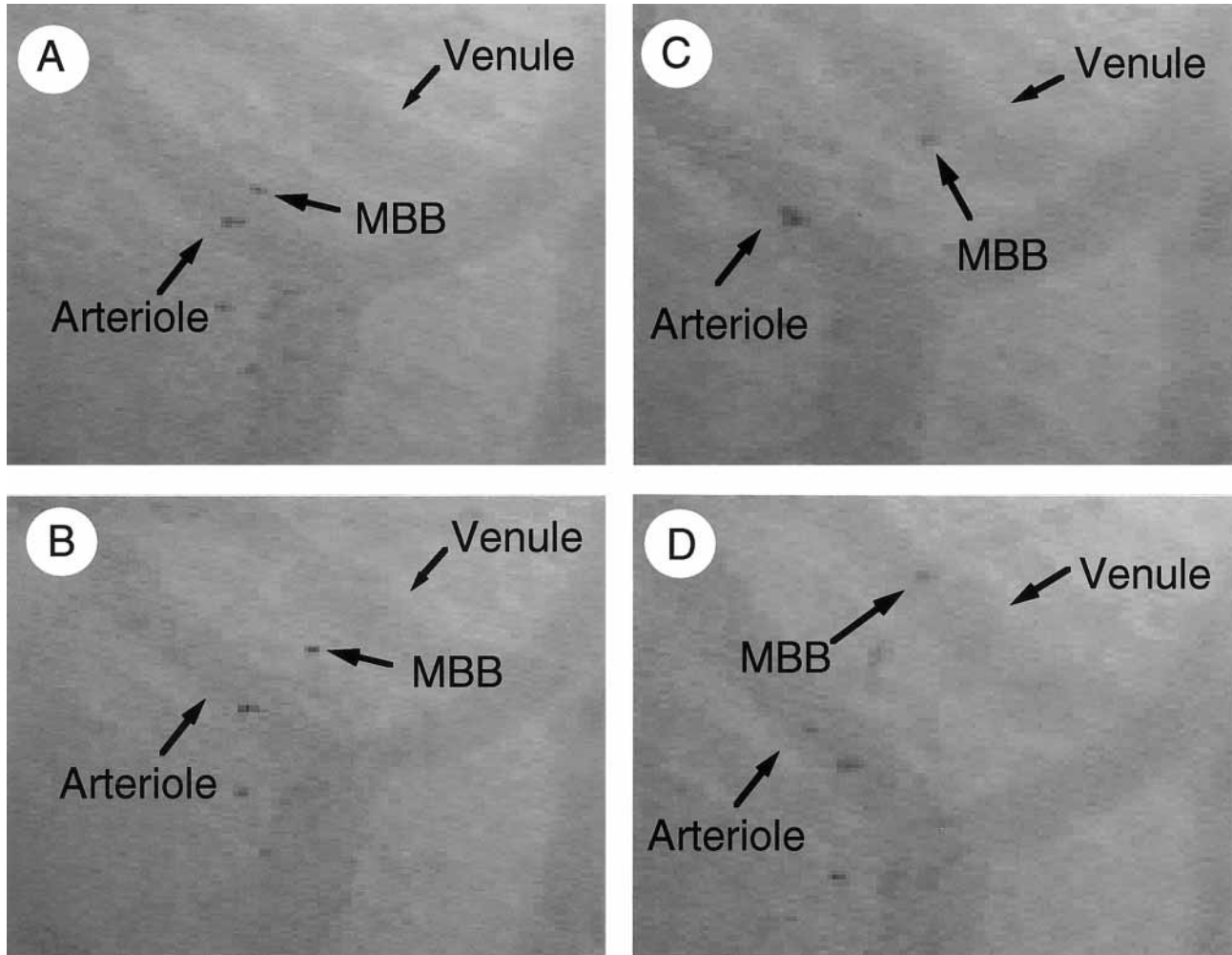
Fig. 5   Intravital observation of arteriovenous (A-V) shunting. Arrows in Figure 5A, 5B, 5C and 5D show movement of the same grain of MBB at 0.07 sec intervals. Figure 5A shows the grain of MBB in an arteriole. In Figure 5B, it has moved into the A-V shunting channel, in Figure 5C into the venule; and in Figure 5D it has flowed along the venule.

Table 1   Detection of A-V Shunting of Blood Flow

	A-V shunting blood flow		total
	positive	negative	
control group	0	15	15
thermal injury group	4*	10	14
total	4	25	29

* $p < 0.05$

larity of venular walls. Ohno *et al.*[31] reported endothelin-dependent venular constriction using intravital microscopy. It was also reported that the level of endothelin-1 in gastric tissue increased 5 hrs after thermal injury in our experimental model.[32] In an ischemia/reperfusion model in the rat mesentery, leukocyte adherence was seen in postcapillary venules, with subsequent elicitation of albumin leakage, and decrease in plasma nitrate/nitrite levels in the superior mesenteric vein were observed.[33] A decrease in the constitutive type of nitric oxide synthase (cNOS) activity in the rat gastric mucosa was also reported in a model of ischemia/reperfusion-induced mucosal injury,[34] and leukocyte-dependent endothelial damage was regarded as a cause of the suppressed nitric oxide production by gastric cNOS. In the present study, we found an increased adherence of leukocytes in venules and exaggerated permeability of venules as indicated by MBB deposition 5 hrs after thermal injury, both of which are also seen in rats subjected to ischemia/reperfusion.[34] One possible cause of the venular irregularity seen in the present study is imbalance between endothelin and nitric oxide. The presence of smooth muscle structures in rat venules was demonstrated using scan-
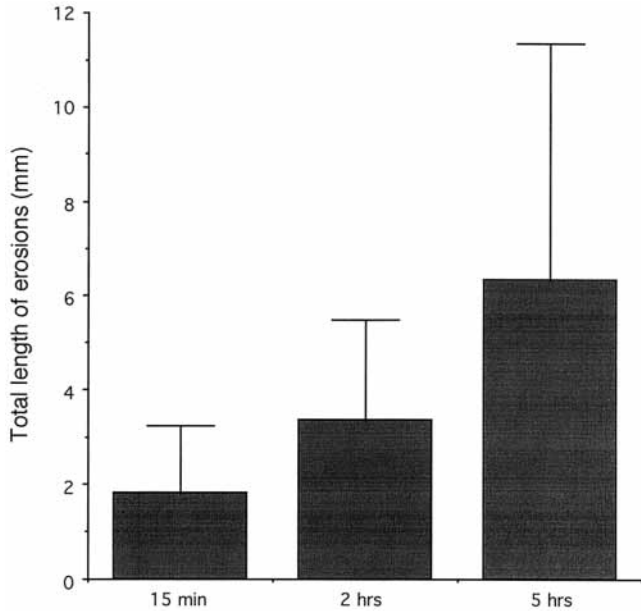
Fig. 6   Total length of erosions. The length of erosions was measured with stereoscopic microscopy and the total length of all erosions per rat was calculated. It increased time-dependently after thermal injury, and the length at 5 hrs was approximately 2 times larger than that at 2 hrs.

ning electron microscopy,[35] suggesting that venules have the ability to contract. However, the presence of muscle structures in vessels smaller than venules have not been reported. We speculated that A-V shunting blood flow develops in a passive manner.

Davenport[36] reported the disruption of the gastric mucosal barrier to hydrogen ions (H^+) as a causative factor in the development of gastric mucosal lesions. Resulting from this "H^+ back-diffusion theory", impairment of gastric mucosal defensive mechanisms is accepted as a cause of gastric mucosal lesions induced by stress. The coexistence of H^+ back-diffusion and microcirculatory disturbance in the stomach after thermal injury was reported in our experimental model,[15] and microcirculation was accepted as an important factor in gastric mucosal defensive mechanisms.

In conclusion, we showed an increase in rolling leukocytes and observed deposits of MBB in venules after thermal injury. Direct intravital observation of A-V shunting blood flow using MBB was also demonstrated. A-V shunting blood flow can be a cause of mucosal hypoperfusion. It is suggested that the microcirculatory disturbance seen 5 hrs after thermal injury contributes to the final step of erosion formation.

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