Differential effects of two doses of aspirin on platelets-vessel wall introduction in vivo.

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Research Article

Platelet cyclooxygenase appears to be more sensitive to aspirin than the arterial endothelial cell cyclooxygenase. To investigate the dose-related effects of aspirin on platelet-vessel wall interaction in acute vascular injury, male New Zealand White rabbits were treated with either (a) aspirin (150 mg/kg body wt; n = 6), (b) aspirin (30 mg/kg; n = 6), or (c) vehicle (n = 10). After treatment, autologous 111In-platelets were injected and deendothelialization of a 10-cm long segment of abdominal aorta was induced by a balloon catheter. Rabbits were killed 3 h after injury and radioactive counts and percentages of injected radioactivity per gram dry weight of tissue or blood were determined. The 30 mg aspirin group had a significantly lower radioactive count (62.13 +/- SD 6.07 x 10(3) cpm) and percentage of injected radioactivity (0.024 +/- 0.003%) per gram dry weight of damaged aortic tissue than the control (1,167.82 +/- 212.31 x 10(3) cpm/g tissue and 0.435 +/- 0.079%, respectively). By contrast, the 150-mg aspirin group had an elevation of radioactive counts (4,343.12 +/- 556.98 cpm) and percentage (1.632 +/- 0.246%) per gram dry weight of damaged tissue. Infusion of exogenous PGI2 was associated with reduction of lesion radioactivity. These findings were supported by ultrastructural findings. Examined under transmission electron microscopy, the injured aortic wall of 30-mg group was covered throughout the segment [...]

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Differential Effects of Two Doses of Aspirin on Platelet-Vessel Wall Interaction In Vivo

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ABSTRACT Platelet cyclooxygenase appears to be more sensitive to aspirin than the arterial endothelial cell cyclooxygenase. To investigate the dose-related effects of aspirin on platelet-vessel wall interaction in acute vascular injury, male New Zealand White rabbits were treated with either (a) aspirin (150 mg/kg body wt; n = 6), (b) aspirin (30 mg/kg; n = 6), or (c) vehicle (n = 10). After treatment, autologous ^{111}In-platelets were injected and deendothelialization of a 10-cm long segment of abdominal aorta was induced by a balloon catheter. Rabbits were killed 3 h after injury and radioactive counts and percentages of injected radioactivity per gram dry weight of tissue or blood were determined. The 30-mg aspirin group had a significantly lower radioactive count (62.13±SD 6.07 × 10³ cpm) and percentage of injected radioactivity (0.024±0.003%) per gram dry weight of damaged aortic tissue than the control (1,167.82±212.31 × 10³ cpm/g tissue and 0.435±0.079%, respectively). By contrast, the 150-mg aspirin group had an elevation of radioactive counts (4,343.12±556.98 cpm) and percentage (1.632 ±0.246%) per gram dry weight of damaged tissue. Infusion of exogenous PGI₂ was associated with reduction of lesion radioactivity. These findings were supported by ultrastructural findings. Examined under transmission electron microscopy, the injured aortic wall of 30-mg group was covered throughout the segment by a single layer of platelets without detectable platelet aggregates, while that of the 150-mg group was diffusely packed with multiple layers of platelets. The findings demonstrate that aspirin (30 mg/kg) prevents platelet aggregate formation at the injured arterial wall, whereas 150 mg/kg promotes platelet thrombus formation.

INTRODUCTION

The inhibitory effect of acetylsalicylic acid (ASA,[1] aspirin) upon platelet aggregation is attributed to its inhibition of platelet cyclooxygenase and a consequent decrease in thromboxane A₂ (TXA₂) production (1). The inhibition involves acetylation of the enzyme and the effect is therefore permanent (2). However, the antiaggregatory effect of ASA upon platelets may be offset by the concurrent inhibition of vascular cyclooxygenase, which leads to decrease in the synthesis of prostacyclin (PGI₂). Recent studies indicate that platelet cyclooxygenase is more sensitive to ASA than the vascular enzyme. This differential sensitivity appears to be related to the rate of acetylation; the rate of cyclooxygenase acetylation in human platelet microsomal preparations is ~60 times higher than that in aorta and coronary artery microsomes (3, 4). Consequently, low doses of ASA selectively inhibit platelet TXA₂ production, whereas high doses inhibit the production of both TXA₂ and PGI₂. The importance of maintaining the balance between these two compounds is underscored by recent animal and in vitro studies that suggest that low doses of ASA are antithrombotic and high doses are thrombogenic (5, 6). To provide more direct evidence in support of this hypothesis, the dose-related effects of ASA on platelet-vessel wall interaction were investigated in a rabbit acute injury model using autologous indium-111 labeled platelets. Our findings indicate that ASA (30 mg/kg) prevents platelet aggregate formation at the injured aortic wall, whereas 150 mg/kg promotes platelet thrombus formation.

METHODS

Acid-citrate-dextrose (ACD) solution (80 mg citric acid, 224 mg anhydrous sodium citrate, and 120 mg anhydrous dextrose

[1] *Abbreviations used in this paper*: ACD, acid citrate dextrose, ASA, acetylsalicylic acid; PGI₂, prostacyclin; TXA₂, thromboxane A₂.

per 10 ml solution) was obtained from E. R. Squibb and Sons, Inc., Princeton, N. J. ACD-saline solution was prepared by mixing one part of ACD with six parts of normal saline. ^{111}In-chloride (2 mCi/ml) was obtained from Mediphysics, Emeryville, Calif. 8-hydroxyquinoline (oxine) and adenosine diphosphate (ADP) were obtained from Sigma Chemical Co., St. Louis, Mo. ASA, a gift of Merck, Sharp and Dohme Research Laboratories, West Point, Pa., was dissolved in 0.1 N NaOH. Collagen, prepared from equine tendons, was obtained from Hormon-Chemie, Munich, West Germany. PGI$_2$ sodium salt was kindly supplied by the Upjohn Co., Kalamazoo, Mich. and was dissolved in 0.05 M tris buffer, pH 9.40 immediately before infusion. It was kept at 4°C throughout the course of infusion. 5F Fogarty catheter was obtained from Edwards Laboratories, Santa Ana, Calif.

Preparation of ^{111}In-labeled platelets. Rabbit platelets were labeled with ^{111}In-oxine by modification of a previously described method (7). Labeling procedures were carried out under sterile conditions. In brief, blood was collected from rabbits by cardiac puncture and divided into two samples: (a) 20 ml was mixed with 4 ml ACD solution and (b) 9 ml with 1 ml 3.8% sodium citrate. Blood samples were centrifuged at 220 g for 10 min to prepare platelet-rich plasma and the remainder further centrifuged at 1,000 g for 20 min to obtain platelet-poor plasma. ACD-platelet-rich plasma was washed twice with 10 ml ACD-saline and then incubated with ^{111}In-oxine complex at room temperature for 20 min. The mixture was centrifuged and the platelet pellet was washed once with 8 ml ACD-platelet-poor plasma to remove residual unbound ^{111}In. The pellet was suspended in 4 ml citrate-platelet-poor plasma. Platelet concentration by phase microscopy and total radioactivity determined by a γ-spectrometer were measured in each sample. Platelet aggregates were examined by phase microscopy in each sample and were undetectable. Platelet aggregation in response to ADP (10 μM) and collagen (10 μg/ml) was normal in the first 10 consecutive animals. As the results were consistent, aggregation was performed once weekly in the subsequent experiments and the results were reproducible. The survival time of the labeled platelets was determined in three normal, nondamaged animals according to the procedure of Heaton et al. (7). Mean initial recovery was 84% and mean survival time based on gamma variate analysis was 3.11 d, which was in keeping with the rabbit normal values reported previously (8).

Deendothelialization of abdominal aorta. Aortic intimal injury was induced by the balloon catheter technique described by Spaet et al. (9). Rabbits were anesthetized with intravenous injection of sodium pentobarbital (25–30 mg/kg). The abdominal aorta was denuded of endothelium by passing a 5F Fogarty catheter into the aorta via the femoral artery. When the tip of the catheter reached the point of aortic bifurcation, the balloon was inflated to a pressure of ~735 mm Hg. While the pressure was maintained, the balloon catheter was passed upward to a level of 10 cm from the bifurcation and down to the original position; this was repeated six times. The balloon was then allowed to collapse, the femoral artery ligated, and the wound closed. The extent of intimal damage was evaluated by infusion of Evans blue (10) in a separate pilot experiment. The intima was diffusely denuded in each of six rabbits. Surgical procedures were performed by the same personnel, and periodic quality control revealed that the endothelium was diffusely denuded for the whole 10-cm segment by this procedure.

Quantitation of platelet accumulation at the vessel wall. At appropriate time intervals after injury, a citrated blood sample was obtained, 1,000 U of heparin was injected intravenously to prevent postmortem clotting and the animal was killed by an overdose of barbiturate. The aorta was sepa-

rated and dissected from the top end of the thoracic aorta just below the arch, to the lower end of the abdominal aorta 1 cm above the bifurcation; which was corresponding to the starting point of balloon catheter injury. Residual tissues attached to the outer surface of the aorta were gently removed. The lower 10-cm injury segment and the upper 5-cm uninjured segment were cut and their radioactivity was determined with a γ-spectrometer. There was no overlapping between the injured and uninjured segments. The segments were then placed in a dessicator and allowed to dry for 48 h. Their weight was then determined. The blood sample that was drawn before killing was similarly treated and the radioactivity and weight were measured. Radioactivity per gram dry weight of tissue or blood was determined and percentage of total injected radioactivity per gram dry weight of tissue was calculated.

Ultrastructure study. Electron microscopic examinations of aortic tissues were carried out in nine additional rabbits that did not receive ^{111}In-platelets. Three animals were in each of the three groups of animals described below. Intimal injury was induced by an identical procedure and at 3 h, 1,000 U of heparin were injected, and the injured and uninjured segments were fixed and examined by transmission electron microscope according to the method previously described (11).

Experiments. All experiments were performed on male New Zealand White rabbits (2–3.5 kg) by using autologous ^{111}In-labeled platelets. A blood sample was drawn for platelet labeling. After the balloon catheter had been placed at the bifurcation, labeled platelets were injected via a marginal vein. The balloon catheter was inflated and intimal damage of a uniformly 10-cm abdominal aortic segment was created. At appropriate time intervals, the animals were killed, and the radioactivity of labeled platelets accumulated at the injured segments, uninjured thoracic aortic segments, and in the blood were determined. A time-course study was initially carried out to select a suitable time period valid for comparative studies. Two animals at each time period were killed at 1, 3, 6, and 24 h after injury. Control animals received a sham operation and were studied at 3 h. Radioactivity per gram dry weight of damaged aortic tissue and blood tended to reduce with time while the radioactivity per gram dry weight of intact aortic tissue remained unchanged over the time periods of study (Table I). 3 h post injury was then selected for the subsequent comparative studies.

To determine the effects of ASA, animals were randomized to three groups: (a) group 1 (n = 6) received ASA 30 mg/kg orally through a gastric tube, (b) group 2 (n = 6) received ASA 150 mg/kg, and (c) group 3 (n = 10) received the aspirin vehicle (0.1 N NaOH). Previous studies in several laboratories have shown that ASA at 10–30 mg/kg selectively inhibits platelet TXA$_2$, while at 150–200 mg/kg it blocks both TXA$_2$ and PGI$_2$-like activities (5, 12). To check whether absorption of ASA had occurred, the blood salicylate level (13) was measured 3 h after aspirin administration in two rabbits of each group; the mean values were 21 mg/dl, 4.3 mg/dl, and 0 for the 150-mg group, the 30-mg group and the vehicle group, respectively. Blood was withdrawn from the rabbits 30 min after the administration of ASA or vehicle. Once the balloon catheter had been inserted, autologous labeled platelets were injected, and intimal damage induced. 3 h following injury, the animal was killed and the lesion radioactivity was determined. Intravenous infusion of PGI$_2$ was carried out in three additional rabbits receiving 150 mg/kg ASA with an infusion pump at a dose of 100 ng/kg per min. Infusion was started 1 h before injury for 2 h. Throughout the infusion period, PGI$_2$ solution was maintained at 4°C in ice.

All the results were expressed as mean±SD. Data were

TABLE I

Time-Course Study of Platelet Deposition on the Damaged Aortic Endothelium

Time intervals after injury	Radioactivity of damaged aortic tissue		Radioactivity of intact aortic tissue		Radioactivity of blood	
h	$\times 10^3$ cpm/g*	Percent‡	$\times 10^3$ cpm/g	Percent	$\times 10^3$ cpm/g	Percent
1	2,114.48	0.601	85.27	0.024	700.65	0.199
3	1,168.06	0.340	72.15	0.021	559.00	0.163
6	999.23	0.263	80.74	0.021	309.28	0.081
24	644.61	0.203	61.99	0.020	192.36	0.065
Sham§	81.37	0.028	67.07	0.023	422.53	0.143

* Counts per minute per gram dry weight of tissue.
‡ Percentage of total radioactivity administered per gram dry weight of tissue. The numbers represent mean values of two experiments.
§ Two animals had sham operations and were killed 3 h after sham operation.

analyzed by a multiple comparison procedure of Dunnett (14, 15), comparing the treatment groups to the vehicle control. The effect of PGI$_2$ on reduction of platelet accumulation at the damaged vessel wall induced by 150 mg/kg ASA was analyzed by Student's t test (16), comparing the PGI$_2$ plus ASA (150 mg/kg) with ASA alone.

RESULTS

All four groups of rabbits were matched for body weight and sex. Radioactivity injected and platelet concentrations in the final platelet suspension and the dry weight of aortic tissues were also matched (Table II). As compared with the control group, the 30-mg ASA group had a significantly lower radioactive count as well as percentage of injected radioactivity per gram dry weight of damaged aortic tissue ($P < 0.01$), while both values were elevated ($P < 0.01$) in the 150-mg ASA group (Table III). Radioactivity of the undamaged segment of thoracic aorta in the 150-mg ASA group was also significantly increased over control values ($P < 0.01$) but no significant difference was noted between the 30-mg ASA group and the control. Radioactive counts as

well as percentages of injected radioactivity per gram dry weight of blood were approximately the same in the control and the treatment groups. The effect of PGI$_2$ infusion upon platelet accumulation at damaged vessel wall in rabbits receiving 150 mg/kg ASA was tested in three additional animals. The data indicate that PGI$_2$ significantly reduced the lesion radioactivity ($P < 0.01$). Radioactivity of undamaged thoracic aorta was also reduced.

Electron microscopic studies of the damaged vessel wall were carried out in nine additional rabbits (three in each group) that underwent similar procedure and treatment except for the omission of ^{111}In-platelets. In all three groups of animals, denudation was diffuse with some areas of medial necrosis and edema. The medial necrosis did not appear to influence platelet accumulation. In the control group, platelet aggregates were frequently noted on the damaged vessel wall, which was covered diffusely by one to two layers of platelets. The damaged vessel wall of the 30 mg/kg group remained covered by a single layer of platelets but aggregates were not encountered. In contrast, the subendothelium

TABLE II

Comparison of Experimental Parameters among Various Groups of Rabbits

	Vehicle control	ASA 30 mg/kg	ASA 150 mg/kg	ASA (150 mg/kg) + PGI$_2$ (100 ng/kg/min)
Number of animals	10	6	6	3
Body weight, kg	2.45±0.12*	2.35±0.23	2.46±0.15	2.39±0.17
Sex	All male	All male	All male	All male
Radioactivity of ^{111}In-platelets, μCi	127±21	122±15	126±14	129±17
Total ^{111}In-platelets ($\times 10^9$) infused	3.25±0.72	3.11±0.59	3.47±0.83	3.27±0.63
Dry weight of injured segments, mg	70.0±21.1	73.4±27.4	70.5±23.3	71.3±20.3
Dry weight of uninjured segment, mg	60.5±28.0	63.2±17.3	70.2±13.4	67.4±19.6

* All the values represent mean±SD.

TABLE III
Dose-Related Effects of Aspirin Treatment on Lesion Radioactivity

Groups	Rabbits	Radioactivity of damaged aortic tissue		Radioactivity of intact aortic tissue	
		$\times 10^3$ cpm/g*	Percent‡	$\times 10^3$ cpm/g	Percent
ASA (30 mg/kg)	6	62.13±6.07§	0.024±0.003§	59.21±16.33	0.023±0.006
ASA (150 mg/kg)	6	4,343.12±556.98‖	1.632±0.246‖	341.44±90.83‖	0.128±0.034‖
ASA (150 mg/kg) + PGI₂	3	1,230.84±769.07¶	0.452±0.283¶	141.43±135.08	0.052±0.050
Vehicle control	10	1,167.82±212.31	0.435±0.079	72.14±39.04	0.027±0.015

* Counts per minute per gram dry weight of tissue.
‡ Percentage of injected radioactivity per gram dry weight of tissue.
§ $P < 0.01$ when compared with control.
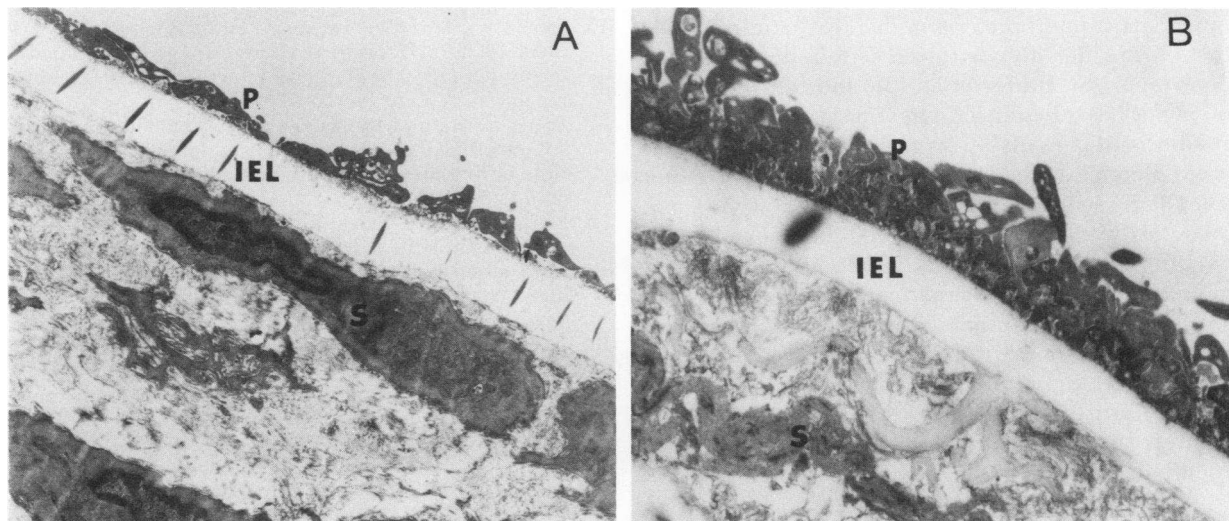‖ $P < 0.01$ when compared with control.
¶ $P < 0.01$ when compared with ASA (150 mg/kg) group.

of the 150-mg/kg group was diffusely packed with multiple layers of platelets (Fig. 1).

DISCUSSION

Indium-111 is regarded as a superior radioisotope for labeling platelets because of its high labeling efficiency and high gamma photon emission (17). These labeled platelets have shown promise in the detection of arterial and venous thrombi in experimental animals and man (18, 19). The present study has used this technique to study the different effects of ASA at 30 mg/kg and 150 mg/kg on platelet-vessel wall interaction in vivo. In the pilot project, we attempted to measure platelet-vessel wall interaction by an external imaging technique as well as by direct measurement of vascular radioactivity. However, the imaging technique, without the use of computer processing proved unsuitable for providing a quantitative discrimination of subtle differences in platelet adherence between the experimental and control animals. On the other hand, the radiometric method described above was reliable for quantifying platelet accumulation at the damaged vessel wall. It has apparent advantages over the ^{51}Cr-labeling technique (20) in that it requires a smaller amount of blood for labeling and it is feasible for external imaging when computer processing is used. It also has an advantage over the morphometric method (21) because it can be used to

FIGURE 1 Representative electron micrographs illustrating the differential effects of 150 mg/kg and 30 mg/kg ASA on platelet adhesion and aggregation on the damaged vessel wall. P denotes platelets, IEL internal elastic lamina and S smooth muscle cell. The vessel wall was covered with a single layer of platelets in an animal receiving 30 mg/kg ASA (A ×4,000). In contrast, the vessel wall of an animal receiving 150 mg/kg ASA was packed with multiple layers of platelets (B ×4,000). Medial edema was observed.

investigate platelet deposition in living experimental animals in which the functional capacity of the vascular system to produce PGI_2 is physiologic. Thus, the ^{111}In-platelet labeling technique should prove to be a valuable tool for evaluating the effects of various prostaglandin inhibitors and/or agonists on platelet thrombus formation.

Our data indicate that 150 mg/kg of ASA promotes the accumulation of platelet aggregates on damaged vessel wall, whereas 30 mg/kg reduces the platelet adherence to only a single layer of platelets. The different effect exerted by these two doses of ASA is likely related to an alteration of PGI_2/TXA_2 balance. The high dose (150 mg/kg) used in the present study has previously been shown to inhibit PGI_2 and TXA_2 production in rabbits (5, 12). The infusion of exogenous PGI_2 into the rabbits treated with 150 mg/kg ASA is consistent with the hypothesis that suppression of local PGI_2 production at the damaged vessel wall contributes to massive platelet aggregate accumulation. It is interesting to note that platelet accumulation at the undamaged endothelium is also increased by ASA (150 mg/kg) and concurrent infusion of PGI_2 tended to reduce the platelet accumulation. On the other hand, the antithrombotic effect of 30 mg/kg ASA is attributed to its selective inhibition of platelet TXA_2 synthesis. It does not inhibit adhesion of a single layer of platelets to damaged endothelium. This may be related to the previous observation that platelet adhesion is more resistant to PGI_2 than platelet aggregation (22).

Our experimental findings in rabbits have certain clinical implications. In the first place, the experimental data support the notion that the antithrombotic effect of ASA lies only within a certain dose range. A recent report by Harter et al. (23) indicates that ASA (160 mg/d) has a beneficial effect on preventing shunt thrombosis in patients undergoing hemodialysis. This dosage is certainly below that used in clinical trials for preventing recurrent myocardial infarction (24) and stroke (25, 26). It has been implied that lack of beneficial effect in the myocardial infarction trial and marginal effect in the stroke trial may be a result of high ASA dosage. Further clinical trials by comparing various doses of ASA are required to resolve this issue. Secondly, that high doses of ASA may promote platelet thrombus formation should arouse some concern about the safety margin of ASA for clinical use as an antithrombotic agent.

ACKNOWLEDGMENTS

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