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BRIEF COMMUNICATION

THROMBOGENIC PROPERTIES OF ULTRA-LOW-DOSE OF ACETYLSALICYLIC ACID IN A VESSEL MODEL OF LASER-INDUCED THROMBUS FORMATION

Christian DOUTREMEPUICH, Omar AGUEJOUF, Dominique PINTIGNY, Marie-Noëlle SERTILLANGES and Odile DE SEZE

Laboratoire d'Hématologie, 3, Place de la Victoire, 33076 Bordeaux cedex, France

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Various studies have demonstrated the antithrombotic effect of high dose acetylsalicylic acid (ASA) and more reports show the similar efficiency of the much better tolerated low dose ASA. Recently, in vitro studies hinted the potent thrombotic properties of ultra-low-dose ASA (ULD-ASA, < 1 mg/day) showing a significant decrease in bleeding time(1,2). This effect was, at least in part, related to the inhibition of endothelial cyclooxygenase -and not to platelet one- leading to a decrease of prostacyclin release (3).

To investigate the effect of ASA in vivo, we used an intravital microscopic technique, allowing to evaluate (anti)-thromboembolic events at previously determined locations of the microvasculature. Thrombus formation was induced by Argon-laser shot. The instrumental test set-up was completed with a video-system. The kinetic feature of thrombus formation was estimated in terms of number of laser shots required to induce endothelium injury leading to thrombosis, number of emboli removed by blood stream and duration of embolization. The reliability and reproductibility of our laser-induced thrombus model have been previously tested in rat mesenteric arterioles and veinules and we showed that ASA used at current doses (50-200 mg/kg) decreased the number of emboli and the duration of embolization (4).

The aim of this preliminary study was the investigation of the effects of ULD-ASA in vivo. In so doing, we evaluated ULD-ASA in our experimental laser-induced thrombus model. This might represent a further step in the understanding of the action of ASA according to the range of concentration used.

Key words : Aspirin, Arterial Thrombosis, Argon Laser, Ultra-low dosage, Embolization.
Corresponding author : Pr. C. Doutremepuich, Laboratoire d'Hématologie, 3, Place de la Victoire, 33076 Bordeaux cedex, France. Phone : 56.92.85.91, Fax : 56.94.31.28.

MATERIALS AND METHODS

Animals

The studies were carried out on male Wistar rats (250-350 g) from Depre Breeding Center (Saint-Doulchard, France). After 8 days of stabling period, the animals were anesthetized with an injection of sodium thiopental (250 mg/kg BW, Nesdonal^R, Specia Laboratories, France) into the femoral muscle. The tested products were then injected subcutaneously in the thorax region. The time corresponding to the administration of the products was noted T_0. The small intestine was then exteriorized through a median laparotomy and the mesentery was spread flat on a glass plate mounted on the microscope table. Investigations were performed in arterioles of the same diameter (40-50 µm) of the fat-free portion of the mesentery. Ten rats were studied in each group. Laser-induced thrombus was induced at T_0+15 min, T_0+35 min and T_0+55 min in three different arterioles of the same rat.

Laser-thrombosis

Vascular lesions were produced by Argon laser (Stabilite 2016, Spectra-Physics, France). The laser beam was coaxially inserted into the inverted light beam path of a microscope (Axiovert 10, Zeiss, France). The wavelength used was 514.5 nm and the energy was adjusted to 120 mW. The exposure time for laser shot was 1/15 sec. Microscopic images were recorded by a videotape recorder through a video camera (DXC 107, Color camera CDD) and were monitored on a television screen (Trinitron color video monitor, PVM 1442 QM, Sony, France). The dynamic course of thrombus formation was continuously monitored and recorded and the time course of the experiment was recorded automatically. A schematic diagram of the complete experimental apparatus has been previously described (4). The laser spot for single shot was positionned on endothelium at the limit between the luminal portion of the vessel wall and the lumen of the arteriole. Three parameters were measured : 1) The number of laser shots required to induce thrombosis was counted (when no visible thrombus occured after the first shot, an another laser shot was applied to the vessel wall at the same site than the first one. The procedure was repeated until a thrombus was visible). 2) The number of emboli removed by the bloodstream after laser injuries. 3) The duration of embolization.

Tested products

Acetylsalicylic acid (ASA) was gently provided by Boiron Laboratories (Sainte-Foy-Lés-Lyon, France). ASA dilution was prepared as follows : 1 g of pure, finely powdered ASA was suspended in 99 ml alcohol (70°); after being vigorously shaken one ml of this dilution was then mixed in 99 ml distilled water and vigorously shaken. This process was repeated up to the last fifteen folds dilution, which contains theoretically 334.10^{-11} molecules/ml corresponding to 10^{-10} nM or about $2.3.10^{-9}$ molecules/kg. An isotonic saline solution (NaCl 0.9%, Delmas perfusion, France) was used as control.

Statistics

Results were expressed as mean $\pm$ Standard Deviation (SD). Statistical analysis was carried out on PCSM^R Software (Deltasoft, France) by Mann-Withney and Kruskal Wallis non-parametric analysis of variance in placebo group and in ASA-treated group taking $p<0.05$ to indicate a significant difference.

RESULTS

The results obtained in control rats and in ultra-low-dose ASA treated rats are summarized in Figure 1. In control 2.7±0.7 laser shots were required to provoke a strong enough endothelium injury to induce a first perceptible thrombus. Thrombus occured within the seconds following the laser shot and grew rapidly. Embolization began within the minute following the laser injury. The number of observed emboli was 1.3±1.6 at T_0+15 min and the embolization continued on 1.9±2.6 min. Then, the thrombus became stable and partially obstructed the vessel lumen. The measures were stopped after 20 min. No significant differences were noted for all these values when thrombus formation was initiated for the second or the third time in another arterioles of the same rat ($p>0.05$, Kruskal Wallis test).

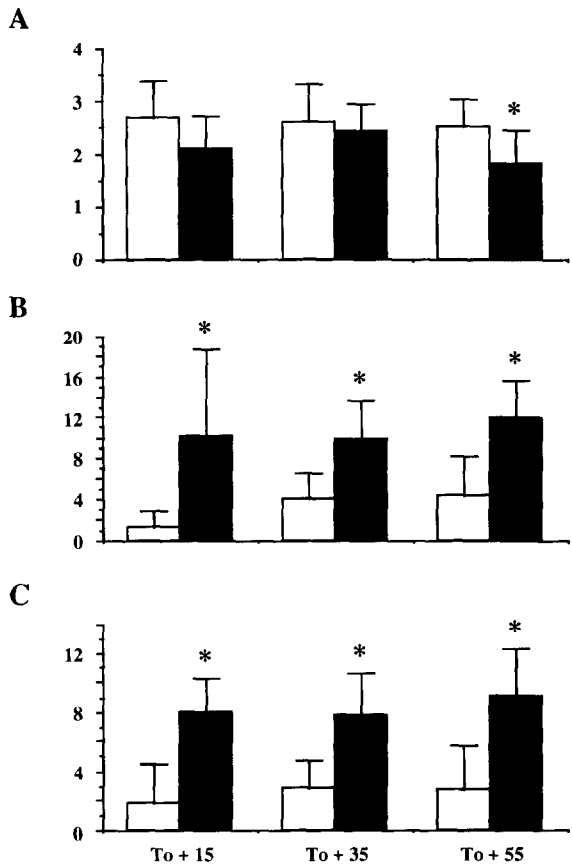


FIG. 1

Decrease in number of laser shots required to induced a thrombus (A), increased in number of emboli (B) and difference in duration of embolization, expressed in minutes (C), after injection of saline buffer (control) or 10^{-10} nM ASA. Laser-lesions were induced 15, 35 and 55 min after ASA injection (To). Results are expressed as the mean ± SD of 10 experiments. ☐ Control ■ ASA
* $p \leq 0.05$ as compared with the control group (Mann-Whitney test).

In ASA-treated rat thrombosis was promoted. The number of laser injuries required to induce thrombus formation was markedly decreased either when the laser shot took place 15, 35 or 55 min after the ULD-ASA administration (334.10^{-11} molecules/ml). However, the level of significance was only reached at T=55 min ($p<0.05$, n=10). The number of generated emboli was significantly increased whatever the laser-injury time used ($p<0.05$, n=10) and the smaller the time the greater the effect. In parallel, duration of embolization was significantly enhanced at all tested times ($p<0.05$, n=10). As in the placebo group, the values of the three parameters obtained for T=35 min and T=55 min were not significantly different from those obtained for T=15 min ($p>0.05$, Kruskal Wallis test).

DISCUSSION

In the present study, we set up an intravital microscopic system allowing the visualization and quantification of arterial thrombotic events in rat. Argon-laser shot provokes the lesion of vessel wall surface and damages endothelial cells scale off. Exposure of deendothelialized surface to circulating blood initiates platelet and coagulation reactions that result in thrombosis. In the initial step, arterial blood flow can disrupt the hemostatic material that accumulated continuously on the thrombogenic luminal surface, and embolization was occurred. Then, the thrombus grew rapidly and obstructed partially the lumen of the vessel.

ASA by its antiaggregant effect plays an antithrombotic role which justifies its use as a prophylactic agent in the prevention of arterial thrombosis (5-7).

In this study, we have investigated the effects of ULD-ASA (334.10^{-11} molecules/ml) in the thrombosis process. Despite difficulty to establish equivalent thrombogeneicity among man and other species, our experimental rat model gives the available evidence showing that the arterial thrombotic events that occure in rat vessels may be proceded in human ones.

Decrease of the number shots required to induce a severe enough endothelial injury leading to thrombus formation indicates that the vessel wall surface is more sensitive to laser action after administration of ULD-ASA.

Previous study (3) has shown that ULD-ASA has no effect on platelets in vitro but acts preferentially on endothelium. However, we cannot exclude the possibility that ULD-ASA can modify the properties of erythrocytes or white cells such as deformability, adhesiveness and reactivity to endothelial cells and/or subendothelium exposed after laser shot. A possible fragility of erythrocytes might lead to the release of proaggregating ADP. Additionally, it is necessary to remember that there are probably as yet unidentified factor(s) which may be influenced by ASA used at ultra-low dosage.

Our results indicate that embolization process was greatly and significantly potentiated after administration of ULD-ASA. A thrombus that has undergone more than the initial phases of organization is not likely to be readily dislodged by the blood stream. The fact that the number of emboli and the duration of embolization were increased after ULD-ASA treatment suggests that emboli showed little or no evidence of organization and could be easily desaggregated. The suceptibility of emboli to thrombosis is consistent with our results obtained for T=35 or 55 min that showed no significant difference

existed in the extent of embolization response when thrombosis was induced for the first, the second or the third time in the same ASA-treated rat.

To confirm this hypothesis, we expected to further investigate thrombosis events in our experimental model. Particularly, determination of the nature of blood cells implicated in the different steps of thrombus formation and detection of the presence of fibrin would give clues to explain the phenomenon.

In conclusion, this performed study demonstrates the prothrombotic efficiency of ULD-ASA in our in vivo thrombosis model in rat arterioles.

Forthcoming studies performed in the two types of vessels with a panel of low and ultra-low dosage of ASA will confirm the phenomenon and will specify where the cleavage between anti- and pro-thrombotic effect of ASA takes place.

Thrombotic properties of ASA may have practical relevance in vivo when ASA is present in the microvasculature at such an ultra-low dose, for example at the end of ASA-therapy or forthward after ASA-administration, in post-operative period. This study well confirms that either dosage regimen or clearance rate of the drug must be taken into account in the prevention of thromboembolism. Thus, potent pro-thrombotic properties of ASA might limit its therapeutic benefit and might be a clue to explain thrombotic complications observed in some ASA-treated patients.

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