A heat shock-related protein, p20, plays an inhibitory role in platelet activation

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Abstract Some low molecular mass heat shock proteins (HSPs) appear to act as molecular chaperones, but their exact physiological roles have not been fully elucidated. We reported previously that a 20-kDa protein (p20), which is classified as a low molecular mass HSP, is present at high levels in skeletal and smooth muscles. In the present study, we investigated a physiological role of p20 on platelet function in vitro and ex vivo. p20 inhibited platelet aggregation using human platelets dose-dependently induced by botrocetin. On the other hand, HSP27, the other type of low molecular mass HSP, did not affect platelet aggregation. When p20 (300 μg/kg) was injected intravenously as a bolus in hamsters, platelet aggregation ex vivo induced by botrocetin was also significantly inhibited. In order to further investigate the inhibitory effect by p20 on platelet activation, we performed platelet aggregation induced by thrombin or ADP using human platelets. p20 markedly prevented platelet aggregation induced by thrombin, but not ADP. These findings suggest that p20 can act intercellularly to regulate platelet functions. Our results may provide the basis for a novel defensive system to thrombus formation.

Key words: Heat shock protein; Platelet; Thrombus

1. Introduction

Heat shock proteins (HSPs), whose expression is induced by many kinds of stress, protect cells from damage under hazardous conditions, and their actions represent one of several defense mechanisms that are operative in vivo. The low molecular mass HSPs are considered to include HSPs with molecular masses of 15–30 kDa. p20 was copurified with αB crystallin and HSP27 from skeletal muscles, and it was identified as a member of the α crystallin low molecular mass HSP family [1,2]. A high level of p20 is formed in normal skeletal and heart muscles, as well as in smooth muscle tissue, and the synthesis of this protein is not induced by heat or chemical stress [3]. Two forms of p20, an aggregated form (p20-L) and a dissociated form (p20-S), were identified in crude extracts of muscles and both forms were purified to homogeneity [3]. p20-S has a dimeric structure with covalent binding via a disulfide bond between subunits. However, p20 redistributes from a cytosolic to an insoluble fraction and dissociates from an aggregated form after heat stress [3]. It has recently been demonstrated that cyclic nucleotide-dependent vasorelaxation is associated with the phosphorylation of this protein [4]. However, the physiological role of p20 has not been clarified. In the present study, we investigated the effect of p20 on platelet function.

2. Materials and methods

2.1. Reagents

A dissociated form (p20-S) of p20, an aggregated form (p20-L) of p20 and HSP27 were purified as described previously [3]. *Bothrops jararaca* (snake toxin) was obtained from Sigma (St. Louis, MO, USA) and botrocetin was purified from *Bothrops jararaca* by Fujimura's methods [5]. The other chemical substances were obtained from Sigma (St. Louis, MO, USA).

2.2. Platelet aggregation using platelet-rich plasma

Human blood was donated from young healthy male volunteers into one-tenth volume of a 3.8% solution of sodium citrate and centrifuged for 12 min at $155\times g$ to obtain platelet-rich plasma (PRP). The concentration of each agonist was chosen to induce about 60% of the maximum aggregation in the control platelets: 2.5 μM ADP or 3.3 μg/ml botrocetin. p20-S, p20-L and HSP27 were preincubated for 20 min with PRP. Platelet aggregation was followed in an aggregometer (Aggrecorder II; DA-3220, Kyotodaiichi-Chemical, Kyoto, Japan) at 37°C with a stirring speed of 800 rpm.

2.3. Platelet aggregation using fixed platelets

In order to further define the inhibitory effect of p20 on platelet aggregation induced by botrocetin, we used fixed platelets [6]. We obtained fixed human platelets as follows: we mixed human PRP with an equal volume of 2.0% paraformaldehyde and incubated the mixture for 30 min at 22°C. The platelets were then isolated by centrifugation at $800\times g$, washed twice with 3.8 mM HEPES-Tyrode's buffer (pH 6.7), and finally suspended in the same buffer (pH 7.4), at a final concentration of 5×10^8 cells/ml. von Willebrand factor (vWF, 10 μg/ml) was added to fixed platelets. Fixed platelets were stimulated by botrocetin after preincubation with both p20-S (1.0 μg/ml) and vWF or in the absence of preincubation for 20 min with both p20-S and vWF. The aggregation induced by 3.3 μg/ml botrocetin was monitored with the aggregometer described above.

2.4. Platelet aggregation ex vivo

Male hamsters (Gold, SLC, Japan) weighing 100–120 g were selected and fed a standard chow (RC4, Oriental Yeast Co., Ltd., Japan). All animal experiments were performed in accordance with institutional guidelines. Hamsters were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and p20-S (30, 100 and 300 μg/kg) was injected as a bolus (300 μl) through the left jugular vein. Blood (4.0 ml each) was collected by heart puncture in sodium citrate (3.15%) 5 min after the bolus injection of p20-S. Platelet aggregation induced by botrocetin (3.0 μg/ml) using PRP was then investigated by the same way as mentioned above.

2.5. Platelet aggregation using washed platelets

We investigated the effect of p20 on platelet aggregation induced by thrombin since thrombin is also a potent agonist for platelet aggregation. Washed platelets were prepared as described previously [6].

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Abbreviations: HSP, heat shock protein; PRP, platelet-rich plasma; vWF, von Willebrand factor; GP, glycoprotein

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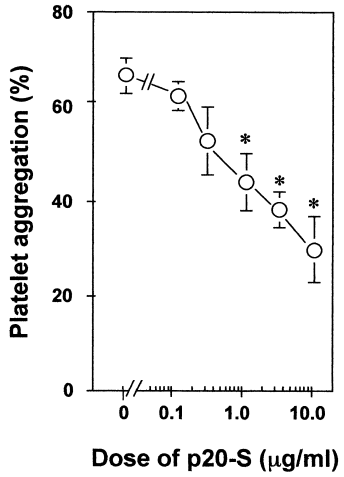

Fig. 1. Dose response inhibition by p20-S of platelet aggregation in human PRP. The aggregation of platelets stimulated by botrocetin (3.0 μg/ml) was performed after preincubation for 20 min with p20-S or vehicle. Data represent the mean ± S.E.M. $*P < 0.01$ vs. control.

Human platelets were counted and adjusted to 4×10^8 cells/ml (final concentration). Washed platelet aggregation was induced by 0.3 unit/ml thrombin after p20-S, p20-L or hsp27 were preincubated for 20 min. Aggregation in response to a stimulant was measured by the aggregometer described above.

2.6. Statistical analysis

All data are expressed as the mean ± S.E.M. The significance of drug effects ($*P < 0.01$) was determined by ANOVA followed by the Student Newman-Keuls test.

3. Results

We did experiments for platelet aggregation induced by botrocetin or ADP using human PRP and investigated the effects of p20-S, p20-L and HSP27 on platelet aggregation. When platelets were preincubated with p20-S for 20 min, p20-S inhibited platelet aggregation dose-dependently induced by botrocetin (Fig. 1), but not ADP. The treatment with p20 in the range between 10 and 100 μg/ml caused similar reduction of platelet aggregation induced by botrocetin. HSP27 and p20-L did not affect botrocetin-induced platelet aggregation.

To define the inhibitory effect of p20-S on platelet aggregation induced by botrocetin in further detail, we performed an experiment using fixed human platelets since glycoprotein

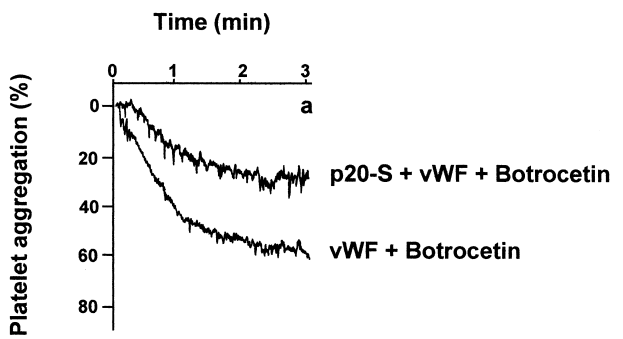

Fig. 2. Inhibition by p20-S of aggregation in human fixed platelets. vWF (10.0 μg/ml) was added to fixed platelets. The aggregation of platelets stimulated by botrocetin (3.3 μg/ml) was performed after preincubation for 20 min with p20-S (1.0 μg/ml) or vehicle.

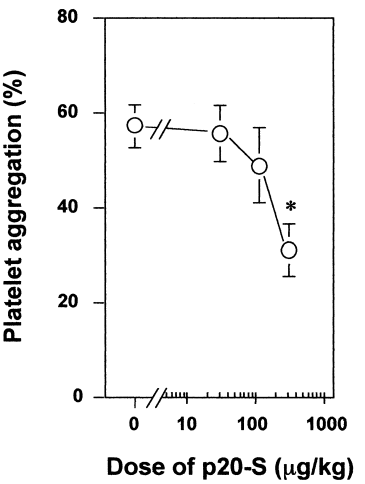

Fig. 3. Dose response inhibition by p20-S of platelet aggregation in hamster PRP. The aggregation of platelets stimulated by botrocetin (3.0 μg/ml) was performed after preincubation for 20 min with p20-S or vehicle. Data represent the mean ± S.E.M. $*P < 0.01$ vs. control.

(GP) Ib of platelet membrane is exposed by treatment with 2.0% paraformaldehyde [6]. Preincubation with p20-S (1.0 μg/ml) inhibited platelet aggregation by 58% as compared with control (Fig. 2). However, in the absence of preincubation with both p20 and vWF, the aggregation of platelets in response to botrocetin was not inhibited.

Additionally, we performed platelet aggregation ex vivo with PRP induced by botrocetin in hamsters. Platelet aggregation was also inhibited by 58% when p20-S at a dose of 300 μg/kg was administered intravenously (Fig. 3). In this case, plasma concentration of p20-S determined at 5 min after an intravenous bolus injection was 8.9 μg/ml by a specific immunoassay as described previously [3] and this level was estimated to be enough for the reduction of platelet aggregation since p20-S at a dose of 3.0 μg/ml caused about 50% inhibition on hamster platelet aggregation in vitro induced by botrocetin (3.3 μg/ml). When we also measured the level of p20

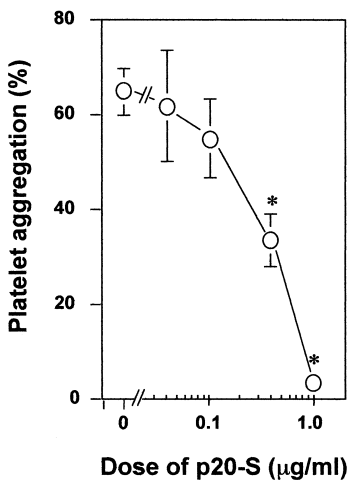

Fig. 4. Dose response inhibition by p20-S of washed platelet aggregation in human. The aggregation of platelets stimulated by thrombin (0.2 units/ml) was performed after preincubation for 20 min with p20-S or vehicle. Data represent the mean ± S.E.M. $*P < 0.01$ vs. control.

in plasma of non-treated hamsters, it showed 4.2 ± 0.8 ng/ml ($n = 4$).

In order to further clarify the role of p20-S on platelet aggregation, we studied the effect of p20-S on platelet aggregation induced by thrombin, a physiological substance in the blood Fig. 4. p20 inhibited platelet aggregation induced by thrombin dose dependently in human washed platelets. However, p20-L and HSP27 did not inhibit platelet aggregation induced by thrombin.

4. Discussion

HSPs are well recognized to protect cellular functions from various damages. However, their physiological roles have not been fully clarified. In the present study, we investigated the effect of p20 on platelet activation, one of the HSPs with low molecular mass, reporting for the first time that p20 inhibits platelet aggregation in vitro.

We investigated the effect of p20 on platelet aggregation in vitro using PRP that was induced by ADP or botrocetin. p20-S inhibited platelet aggregation induced by botrocetin, but not ADP. p20-L or HSP27 did not prevent platelet aggregation. We speculated that an aggregated form of p20 (p20-L) was inactive and that p20-L was dissociated by some kind of stimulation and changed to p20-S, an active form, for effect on platelets.

Botrocetin only stimulates the binding of vWF to platelet GPIb, and then induces platelet aggregation. Therefore, it is possible that the inhibition of platelet activation by p20-S is related to the prevention of the vWF-GPIb axis. Additionally, the result of an experiment using fixed platelets showed that platelet aggregation induced by botrocetin was not observed in the absence of preincubation by either p20-S or vWF. This finding indicates that p20-S might interact with vWF but not with platelet GPIb. Finally, we performed platelet aggregation ex vivo using hamsters since the behavior of hamster platelets is similar to human platelets [7–9]. An intravenous injection of p20-S showed a dose dependent antiplatelet effect. Therefore, our findings make us speculate that p20 might play an inhibitory effect on platelet activation in vivo.

However, botrocetin is not a physiological agonist in blood circulation and we additionally investigated the effect of p20 on platelet aggregation induced by thrombin. Thrombin is well known to be a potent agonist for platelet aggregation and recently interaction between thrombin and GPIb on platelets has been reported and the fact that GPIb functions as the high-affinity platelet receptor for thrombin has been shown [10–12]. Indeed p20-S completely prevented the platelet aggregation induced by thrombin. These findings indicated that p20 might have an important physiological role in blood circulation. Moreover, in the clinical setting, plasma levels of p20 in patients with acute dissecting aneurysm (0.66 ± 0.06 ng/ml, $n = 3$) were higher than those of healthy subjects (< 0.06 ng/ml, $n = 4$). This observation also indicates that p20 actually plays a role in physiological conditions in human. The results promise a potential usefulness of p20 as antiplatelet strategy.

In our experiments, p20 did not prevent ADP-promoted platelet aggregation. ADP stimulates platelets via its specific receptors on platelet membrane and this pathway is different from that of botrocetin [13,14]. Therefore it seems unlikely that p20-S interfered with platelet activation via ADP receptors.

To the best of our knowledge, the present report is the first to describe the function of p20 on platelet activation. In conclusion, these findings suggest that p20 can act intercellularly to regulate platelet functions. Our results may provide the basis for a novel aspect of a defensive system to thrombus formation.

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