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Possible Involvement of Prostaglandin(s) and cAMP, not NO/cGMP, in the Mechanism of Carp Thrombocyte Aggregation

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Aggregation of carp (*Cyprinus carpio*) thrombocytes, in comparison with that of rat platelets, was studied by the whole blood method using an impedance aggregometer. Carp thrombocyte aggregation, much like that of rat platelets, was triggered by collagen. Adenosine 5'-diphosphoric acid (ADP) caused rat platelet aggregation but failed to induce carp thrombocyte aggregation. While arachidonic acid effectively induced rat platelet aggregation, carp thrombocytes did not respond to the addition of arachidonic acid in 3 out of 4 cases. These findings show differences in aggregation behaviors, in response to various aggregating agents, between rat platelets and carp thrombocytes. The aggregating responses of rat platelets and carp thrombocytes induced by collagen were inhibited by indomethacin, a typical cyclooxygenase inhibitor. This raised the possibility of prostaglandin(s) involvement in the mechanism of carp thrombocyte aggregation, in a manner similar to that in rat platelets. Thrombocyte aggregation, induced in the carp by collagen, was inhibited by stable prostacyclin (PGI_2) analogues (Iloprost and Beraprost), 3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor) and N^6, O^2-dibutyryl adenosine 3': 5'-cyclic monophosphoric acid (db-cAMP), as similar to that of rat platelet aggregation. These results raised the possibility of cyclic AMP (cAMP) involvement in the mechanism of carp thrombocyte aggregation, in a manner similar to that in rat platelets. Thrombocyte aggregation, induced in the carp by collagen, failed to be inhibited by sodium nitroprusside (SNP), a typical nitric oxide (NO) releaser, while SNP partially but significantly inhibited collagen-induced platelet aggregation in the rat. This suggests that NO/cyclic GMP (cGMP) does not play an inhibitory role in the mechanism of carp thrombocyte aggregation, unlike the aggregation in rat platelets.

Keywords : Carp, Thrombocyte Aggregation, Collagen, Prostaglandin, cAMP

Introduction

In mammals, platelet aggregation plays a role in hemostasis physiologically to stop bleeding[1]. However, excessive aggregation of platelets amplified pathologically induces thrombosis[1]. Therefore, mechanism of platelet aggregation has been studied in human and mammals extensively and drugs inhibiting platelet aggregation have been developed[1]. Fish thrombocyte, whose equivalent is mammal's platelet, is known to take part in inhibition of bleeding, physiologically[2], but the mechanism for thrombocyte aggregation in fish has not been studied compared with mammal's case.

In the present study, we tried to clarify a part of mechanisms of fish thrombocytes aggregation, using carp thrombocytes, in comparison with that of rat platelets. The main questions are as follows : 1) Are there any differences in aggregation behaviors, in response to various aggregating agents such as collagen, arachidonic acid, and ADP ? 2) Prostaglandin(s), cAMP and NO/cGMP are typical signal transmitters in mammalian cells[1]. Are they involved in the mechanism of carp thrombocyte aggregation?

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Methods

Aggregation of carp (*Cyprinus carpio*) thrombocytes, in comparison with that of rat platelets, was studied by the impedance method with whole blood aggregometer[7,8]. Simplified illustration of the whole blood aggregometer (type 560-VS ; Chrono-log Co., Haverton, PA, USA) is shown in Fig. 1. The blood (1000 μl) in the cuvette, added by one-nineth volume of 3.8% sodium citrate and kept from clotting, is inserted with two electrodes and weak electric current is flowing between two electrodes. Before the aggregation, platelets or thrombocytes are dispersed uniformly in the blood. The blood is pre-incubated with 3 mM $CaCl_2$ (added) for in the presence of inhibitor or its vehicle, and then aggregating agent is added to induce aggregation. After the aggregation, aggregates of platelets or thrombocytes are attached to the electrodes. Therefore, electric current is disturbed and the impedance of the blood is increased after the aggregation. The change of impedance is recorded continuously and the aggregation curve is obtained. The extent of aggregation is expressed as the maximum change of impedance. The temperature of aggregation reaction in carp and rat are 25 and 37 degrees centigrade, respectively.

Results and Discussion

Some differences in aggregation behaviors, in response to various aggregating agents such as collagen, arachidonic acid, and ADP

Rat platelet aggregation was induced by 1-10 μg/ml of collagen. Similarly, carp thrombocyte aggregation was also triggered by 1-10 μg/ml of collagen. Arachidonic acid (10-100 μM) effectively induced rat platelet aggregation. Meanwhile, carp thrombocytes did not respond to the addition of 100 μM of arachidonic acid in 3 out of 4 cases. Adenosine 5'-diphosphoric acid (ADP ; 6-60 μM) effectively caused platelet aggregation in rat. However, 60 μM of ADP failed to induce carp thrombocyte aggregation. Using the same blood sample, collagen clearly caused carp thrombocyte aggregation. Therefore, signal transduction system stimulated with ADP might be lacking in the case of carp thrombocytes. These findings show some difference in aggregation behaviors, in response to various aggregating agents, between rat platelets and carp thrombocytes (Table 1). The common aggregating agent was shown to be collagen. Therefore, in the next step, we examined the mechanism of collagen-induced carp thrombocyte aggregation using

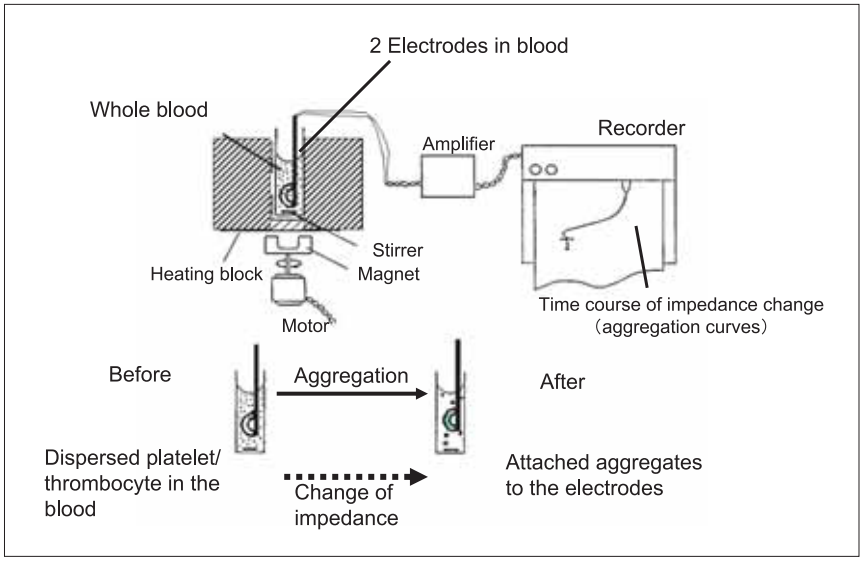

Fig. 1. Impedance method with whole blood aggregometer for measurement of platelet (thrombocyte) aggregation

Table 1. Comparison of aggregation behaviors of rat platelets and carp thrombocytes (+ : aggregation, − : no effect, ± : uncertain)

	Collagen	Arachidonic acid	ADP
Rat	+	+	+
Carp	+	±	−

several inhibitors in comparison with rat platelet.

Kayama et al.[9] reported similar results for aggregation behaviors of carp thrombocytes to collagen, arachidonic acid and ADP using the experimental method under microscope watching aggregation of washed thrombocytes.

Possible involvement of prostaglandin(s) in the mechanism of carp thrombocyte aggregation.

Prostaglandins, metabolites of arachidonic acid cascade, are typical signal transmitters in mammalian cells including platelet. The next question is whether prostaglandins are involved in the mechanism of carp thrombocyte aggregation induced by collagen.

Arachidonic acid is released from glycerophospholipids of cell membrane after stimulation of the cell. Released arachidonic acid is metabolized by cyclooxygenase to produce prostaglandin H_2 (PGH_2). Various prostaglandins are generated from PGH_2. Thromboxane A_2 (TXA_2) is known to be involved in the mechanism of platelet aggregation in mammals[10]. Indomethacin (Fig. 2) is a typical cyclooxygenase inhibitor[11]. If the aggregation is suppressed by the pretreatment of thrombocytes with indomethacin, prostaglandin(s) might be involved in the mechanism of aggregation.

The aggregating response of carp thrombocytes induced by collagen (1 μg/ml) was inhibited by indomethacin (3-30 μM), as similar to rat platelet (Table 2). This raised the possibility of prostaglandin(s) involvement in the mechanism of carp thrombocyte aggregation, in a manner similar to that in rat platelets. Hill et al.[12] also reported the involvement of prostaglandin(s) in the mechanism of thrombocyte aggregation in rainbow trout using washed thrombocytes.

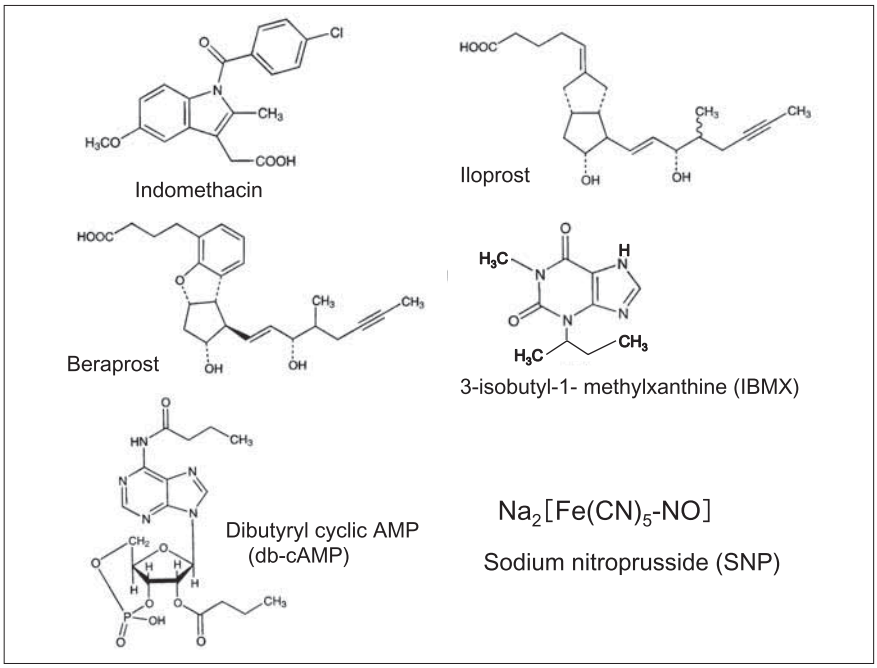

Fig. 2. Chemical structures of the pharmacological reagents used in the present study

Table 2. Effects of indomethacin, cAMP related comounds and SNP on the aggregations of rat platelets and carp thrombocytes induced by collagen

	Collagen-induced aggregation	
	Rat platelet	Carp thrombocyte
Indomethacin	Inhibition	Inhibition
PGI_2 analogues IBMX db-cAMP	Inhibition	Inhibition
SNP	Inhibition	No effect

Possible involvement of cyclic AMP (cAMP) in the mechanism of carp thrombocyte aggregation.

cAMP is one of the most important signal transmitters in mammalian cells and is related to various cellular functions. Another question is whether cAMP is involved in the mechanism of carp thrombocyte aggregation induced by collagen. In mammals, it is known that cAMP plays a role in inhibitory regulation of the platelet aggregation[13]. cAMP in the cells is elevated by the activation of adenylyl cyclase or the inhibition of phosphodiesterase. Iloprost and Beraprost are stable prostacyclin (PGI_2) analogues and it has been reported that they bind to PGI_2 receptor on the cell membrane resulting an activation of adenylyl cyclase[14,15]. It is also reported that 3-isobutyl-1-methylxanthine (IBMX) inhibits phosphodiesterse resulting to increase intracellular cAMP level[16]. In addition, it is reported that dibutyryl cyclicAMP (db-cAMP), which is a cell membrane permeable cAMP analogue, can cause the same effect to cAMP[17]. The chemical structures of cAMP equivalent analogue and intracellular cAMP elevating agents used in our study are shown in Fig. 2.

Thrombocyte aggregation, induced by collagen (1 μg/ml) in carp, was inhibited by Iloprost. The inhibitory effect of Iloprost was concentration-dependent and significant at 1 and 3 μM. An almost similar result was obtained in rat platelet aggregation. Thrombocyte aggregation, induced by collagen (1 μg/ml) in carp, was inhibited by Beraprost. The inhibitory effect of Beraprost was concentration-dependent and significant at 0.1 and 1 μM. An almost similar result was obtained in rat platelet aggregation. Thrombocyte aggregation, induced by collagen (1 μg/ml) in carp, was inhibited by IBMX. The inhibitory effect of IBMX was concentration-dependent and significant at 0.1 and 0.3 mM. An almost similar result was obtained in rat platelet aggregation. Thrombocyte aggregation, induced by collagen (1 μg/ml) in carp, was inhibited by db-cAMP. The inhibitory effect of db-cAMP was concentration- dependent and significant at 10 mM. An almost similar result was obtained in rat platelet aggregation. In our study, either Iloprost and Beraprost or IBMX inhibited thrombocyte aggregation in carp thrombocyte as similar to rat platelet (Table 2). In addition, the carp thrombocyte aggregation was also inhibited by db-cAMP (Table 2). Therefore, these results clearly suggest that cAMP is an inhibitory mediator of carp thrombocyte aggregation, as in the case of mammalian platelets (Table 2).

The reports that discuss the role of cAMP in fish's blood cells are very few. It has been reported that, in fish erythrocytes, Na^+/H^+ exchange and sodium permeability were controlled by elevating cAMP level after activation of adenylyl cyclase by β-adrenergic stimulation or forskolin[18-20]. Any report that discusses a role of cAMP in fish's thrombocyte function has not been found.

Possible lacking of cyclic GMP (cGMP) involvement in the mechanism of carp thrombocyte aggregation

cGMP is another signal transmitter in the cell as well as cAMP, and plays a role in the mechanism of platelet aggregation in mammal[13]. cGMP is enzymaticaly synthesized by the activation of guanylyl cyclase mediated by the

nitric oxide (NO) which is generated in the endothelial cells of blood vessels[21]. On the other hand, cGMP can be synthesized by the addition of the chemicals, called NO releasers, such as sodium nitroprusside (SNP) (Fig. 2) which was decomposed in blood and releases NO[22]. This NO can also activate guanylyl cyclase and produce cGMP[22]. Therefore, NO releasers such as SNP are often used as a research tool to examine an involvement of NO/cGMP system in the mechanism of cell function.

Platelet aggregation, induced by collagen (1 μg/ml) in rat, was inhibited by SNP. The inhibitory effect of SNP was concentration-dependent. The inhibition of platelet aggregation at 3 and 10 mM was partial but statistically significant. Meanwhile, thrombocyte aggregation, induced by collagen (1 μg/ml) in carp, failed to be inhibited by SNP up to the concentration of 10 mM. There observed clear difference in inhibitory effects of SNP on the aggregation between rat and carp (Table 2). This suggests that NO/cGMP system does not play an inhibitory role in the mechanism of carp thrombocyte aggregation, unlike the aggregation in rat platelets (Table 2).

There have been no reports studying the involvement of NO/cGMP in the mechanism of fish thrombocyte aggregation. Therefore, our study might be the first case indicating that there are clear difference of the involvement of NO/cGMP system between thrombocyte aggregation in fish and platelet aggregation in mammal.

Similar examination for the involvement of NO/cGMP system in the regulation of the blood circulation in carp was reported by Park et al.[23]. In rat, SNP, which generates NO immediately, or Bradykinin and histamine, that synthesize NO indirectly by the stimulation of the endothelial cells, effectively caused the relaxation of blood vessels, while, in carp, they failed to cause the relaxation[23]. Moreover, SNP increased in cGMP level of blood vessels in rat, but not in carp[23]. Therefore, the findings of Park et al.[23] suggested that NO/cGMP system was not involved in the mechanism blood vessels'relaxation in carp as similar to our findings of carp thrombocyte aggregation.

The participation of the NO/cGMP system in the blood circulation has also been examined in rainbow trout[24-26], eel[27, 28], sharks (*Squalus acanthias*)[29], and icefish (*Chionodraco hamatus*)[30]. These reports indicated that, in some cases, NO, synthesized in endothelial cells, and cGMP were taking part in the regulation of blood vessel relaxation and blood pressure[24, 26, 27, 29, 30], as similar to mammals. However, in the same fish, NO/cGMP system was not involved in such mechanism under the other experimental conditions[25, 27-29]. Therefore, the interpretation was complicated and there were many points to be cleared.

Conclusion

1. There are some differences in aggregation behaviors, in response to some aggregating agents (collagen, arachidonic acid and ADP), between rat platelets and carp thrombocytes.
2. Prostaglandin(s) and cAMP are possibly involved in the mechanism of carp thrombocyte aggregation induced by collagen, in a manner similar to that in rat platelets.
3. NO/cGMP may not play an inhibitory role in the mechanism of carp thrombocyte aggregation induced by collagen, unlike the aggregation in rat platelets.
4. Schematic mechanisms of rat platelet aggregation and carp thrombocyte aggregation induced by collagen and the effects of indomethacin, cAMP related comounds and SNP on the aggregation are shown in Fig. 3 and Fig. 4.

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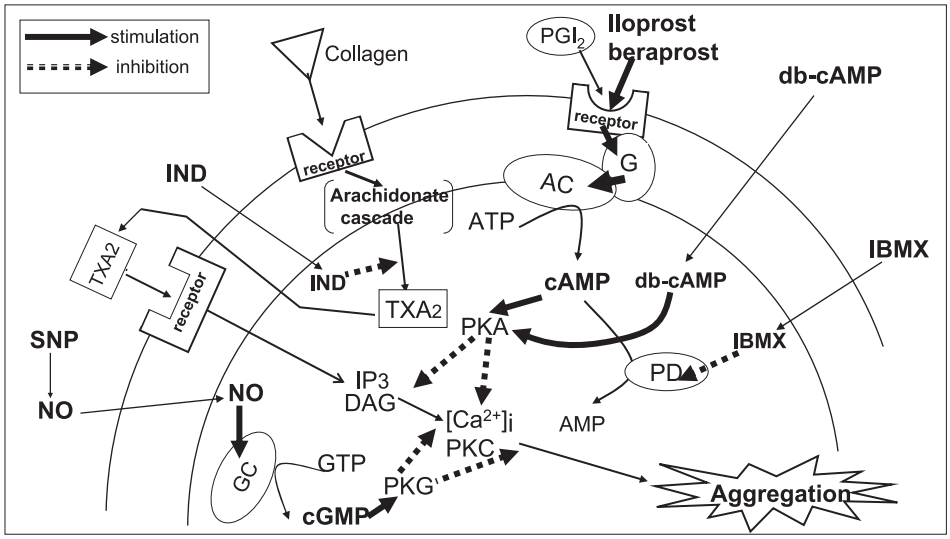

Fig. 3. Schematic mechanism: Inhibition of collagen-induced platelet aggregation in rat by IND, cAMP related compounds and SNP
Abbreviations used are as follows: IND: indomethacin, PGI_2: prostacyclin, TXA_2: thromboxane A_2, IMBX: 3-isobutyl-1-methylxanthine, db-cAMP: dibutyryl cyclic cAMP, G: G protein, AC: adenylyl cyclase, PD: phosphodiesterase, PKA: protein kinase A, PKC: protein kinase C, IP_3: inositol triphosphate, DAG: diacyl glycerol, $[Ca^{2+}]_i$: intracellular free Ca^{2+}, SNP: sodium nitroprusside, NO: nitrogen oxide, GC: guanylyl cyclase, PKG: protein kinase G

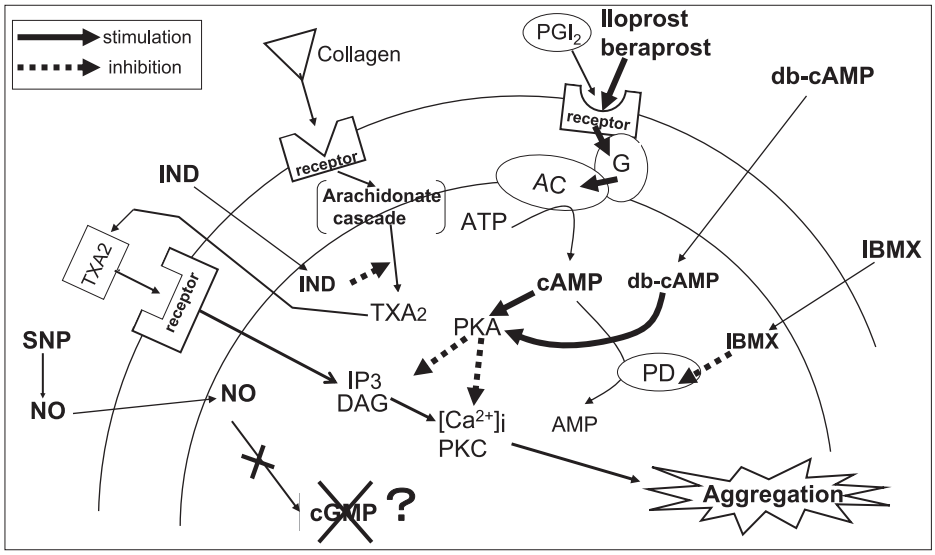

Fig. 4. Schematic mechanism: Inhibition of collagen-induced thrombocyte aggregation in carp by IND, cAMP related comounds and not by SNP
Abbreviations used are as same as Fig. 3.

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