α_1 -Acid Glycoprotein Suppresses Rat Acute Inflammatory Paw Edema through the Inhibition of Neutrophils Activation and Prostaglandin E_2 Generation

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α_1 -Acid glycoprotein (AGP) is an acute phase protein. Whereas the expression of AGP in an inflammatory state is enhanced by inflammatory cytokines including interleukin-1, 6 (IL-1 and IL-6), and tumor necrosis factor- α (TNF- α), the biological significance of AGP remains unclear. In the current study, the anti-inflammatory effect of AGP on the acute inflammatory state was examined *in vivo* and *in vitro*. AGP suppressed carrageenan-, dextran- and kaolin-induced paw edema and vascular permeability in rat. These results suggest that both initial inflammatory mediators (serotonin and histamine) and later inflammatory mediators (prostaglandin and bradykinin) are involved in the anti-inflammatory effects of AGP. In fact, prostaglandin E_2 (PGE $_2$) generation in plasma was significantly inhibited by AGP. Moreover, AGP inhibited the migration of neutrophils treated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) through membrane filter. In addition, AGP significantly suppressed superoxide generation from neutrophils that has been treated with fMLP or phorbol 12-myristate 13-acetate. These results imply that the anti-inflammatory effect of AGP may involve the inhibition of neutrophils migration. The data obtained in this study support a scenario in which an increase in AGP concentration in pathological conditions suppresses inflammation reactions induced by autacoids and neutrophils activities and that AGP plays an important role in the maintenance in the body.

Key words α_1 -acid glycoprotein; paw edema; neutrophil; prostaglandin E_2

α_1 -Acid glycoprotein (AGP), a member of the lipocalin family, is a polypeptide containing 2 disulfide bonds and 5 carbohydrate chains, the latter of which account for about 40% of its total mass of 40 kDa.^{1–4)} The half-life of ¹²⁵I-labeled AGP is approximately 5 d.⁵⁾ Whereas plasma AGP levels are in the range of 0.5–1.0 g/l,⁶⁾ AGP is known to be an acute phase protein, in that its level increases several-fold during burn, trauma or infection.^{7,8)} The expression of AGP is controlled by a combination of major regulatory mediators, *i.e.* glucocorticoids and a cytokine network involving interleukin-1, 6 (IL-1 and IL-6) and tumor necrosis factor- α (TNF- α).⁶⁾ AGP is mainly synthesized and secreted from liver. On the other hands, it is known that monocytes, lymphocytes and endothelial cells could also secrete AGP.⁹⁾ In addition, fluorescence microscopy studies indicate that AGP is present at an inflammatory site.^{10,11)} Therefore, AGP may, to some extent, be involved in the inflammatory event. It is noteworthy that AGP has been reported to inhibit platelet aggregation,^{12,13)} suppress proliferative response in lymphocyte cultures induced by anti-CD3¹⁴⁾ and phytohemagglutinin,¹⁵⁾ and increase the secretion of an IL-1 inhibitor by murine macrophages, most likely the IL-1 receptor antagonist.^{16,17)} Sialyl Lewis X (sLe^X) is the ligand for the cell adhesion molecules E-selectin and P-selectin, which are involved in the inflammation-dependent adhesion of neutrophils, monocytes, or T-cells to endothelial cells or platelets.¹⁸⁾ The sLe^X form of AGP is induced during inflammation.¹⁹⁾ It has also been reported that AGP inhibits the sLe^X/E-selectin interactions.²⁰⁾ Furthermore, AGP appears to be involved in the maintenance of the capillary permeability barrier.²¹⁾ It has been proposed that this effect is brought about by increasing the negative charge of the capillary endothelium,^{22,23)} thereby, reducing

the transvascular transport of polyanionic macromolecules.^{21,22)} However, its exact biological function *in vivo* and mechanism of action remain obscure. Detailed information on its anti-inflammatory effects would be useful in terms of elucidating the role of acute phase proteins that are produced at an accelerated rate in body during an acute phase response.

In the present study, we reported on a detailed investigation of the anti-inflammatory effects of AGP. One of the major consequences of acute inflammation is leakage of plasma elements from blood vessel to injured regions and the infiltration of neutrophils. Anti-inflammatory effects *in vivo* involved a determination of whether AGP suppressed carrageenan-, dextran- or kaolin-induced paw edema and vascular permeability. We also elucidated the effects of AGP on the chemotactic response of neutrophils to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and on superoxide anion generation in response to fMLP and phorbol 12-myristate 13-acetate (PMA). Moreover, the effects of AGP on prostaglandin E_2 (PGE $_2$) generation were examined.

MATERIALS AND METHODS

Materials Human AGP (purified from Cohn fraction VI), human serum albumin (HSA), lambda carrageenan, formamide, arachidonic acid, PMA and fMLP were purchased from Sigma Chemical Co (St Louis, MO, U.S.A.). Dextran, kaolin, Evans blue and acetylsalicylic acid was obtained from Nacalai Tesque (Kyoto, Japan). LymphoprepTM was obtained from Axis-Shield PoC AS (Oslo, Norway). PGE $_2$ immunoassay kit was obtained from Funakoshi Co., Ltd (Tokyo, Japan). All other materials were of reagent grade, and all so-

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lutions were prepared using deionized, distilled water.

Desialylation of AGP Human AGP was desialylated enzymatically, using methodology described by Primožic and McNamara,²⁴ using an immobilized neuraminidase obtained from *Clostridium perfringens*. Human AGP (40 mg) was dissolved in 5 ml of 67 mM phosphate buffer (pH 7.4) and 2 units of enzyme were added. The mixed solution was incubated at 37°C, with gentle stirring at 60 rpm for 24 h. After incubation, the mixture was centrifuged and filtered to remove the immobilized enzyme. A small part of the filtrate was used for measuring sialic acid by the thiobarbituric acid method.²⁵ The product was dialyzed against deionized water and the dialysate lyophilized. Approximately 95% of sialic acid was removed, leaving an average of one sialic acid residue per protein molecule. The mol. wt of asialo-AGP was therefore 40000.

Measurement of Paw Edema Male Sprague-Dawley rats (110–130 g) were used as experimental animals. 1% w/v carrageenan, 1% w/v dextran and 20% w/v kaolin in 0.1 ml saline were injected subcutaneously into the plantar surface of right hind paw of rats at time zero. The paw volumes, up to a fixed mark at the level of lateral malleolus, were measured by recording the time-dependent volume displacement of a water column. AGP (45 μ mol/rat) or HSA (45 μ mol/rat) was given by tail vein injection 1 h before injection of the inflammatory agents injection and the edema volume was compared with control group in a time-dependent manner.²⁶

Measurement of Vascular Permeability Extravasation of plasma albumin was measured by the Evans blue leakage method. Evans blue (60 mg/kg) was given by tail vein injection and acute inflammation was induced 30 min later. Rats were killed by exsanguination 1 h, 3 h or 8 h after the application of carrageenan, dextran or kaolin respectively. The paws were removed and minced. The minced paws were then incubated with formamide (2 ml) for 18 h at 37°C. The solution was subsequently filtered and the optical density of the filtrate measured at 620 nm in a Jasco Ubest-35 spectrophotometer. The amount of the accumulated Evans blue was expressed as mg of dye/g of wet tissue.²⁷

Isolation of Rat Neutrophils Blood was collected in heparinized tubes. A 6% dextran solution was added to the blood and the supernatant was collected after 1 h. The equal parts of LymphoprepTM and the supernatant were mixed. After centrifugation at 1700 rpm for 30 min, the sediment (neutrophils) was collected. If the sediment contained red blood cells, they were removed by hemolysis. The sediment was washed two times with Hanks' balanced salt solution (HBSS) by repeated resuspension and then centrifuged at 300 rpm for 10 min. Neutrophils (10⁷ cells/ml) were prepared in HBSS. Cell viability was assessed using the trypan blue exclusion test.

Chemotaxis Assay Chemotaxis was determined using a chemotaxis chamber (Ieda Trading Co.). Twenty-four microliters of neutrophils (10⁷ cells/ml) and AGP (45 μ M) or HBSS (control) were placed in the upper chamber and 29 μ l of chemotactic factor (fMLP 10⁻⁷ M or PMA) in the lower chamber. The plates were incubated at 37°C in humidified 95% air, 5% CO₂ for 3 h. Migration was measured as number of neutrophils that passed into the lower chamber through a polycarbonate membrane filter with a pore size of 3 μ m.

Chemiluminescence Assay Chemiluminescence was measured using a luminometer (Lumat LB9507, EG&G BERTHOLD) with disposable polypropylene tubes. Neutrophils (10⁷ cells/ml) incubated for 10 min with AGP (45 μ M) or HBSS (control) were added to luminol and stimulated with fMLP (10⁻⁷ μ M) or PMA (μ M) at time zero. The final volume of the reaction mixtures was adjusted to 200 μ l.

Determination of PGE₂ Generation AGP (10 mg/rat) or HSA (15 mg/kg) in 0.1 ml saline was administered by tail vein injection 1 h before the tail vein injection of arachidonic acid (30 mg/kg) in rats. Blood was collected 1 min after the injection of arachidonic acid. After centrifugation at 3000 rpm for 10 min, plasma was collected. PGE₂ in the plasma was determined using a PGE₂ immunoassay kit.

RESULTS

In Vivo Studies for the Anti-inflammatory Activities of AGP Carrageenan-induced paw edema, which shows dramatic leucocytic migration, is mediated by histamine and serotonin in the initial stage, and prostaglandin and bradykinin in the later stage.²⁸ Dextran-induced paw edema is activated by histamine and serotonin without leucocytic migration.²⁹ Kaolin-induced paw edema is mediated by prostaglandin and bradykinin.³⁰ As shown in Figs. 1A–F, AGP and asialo-AGP suppressed carrageenan-, dextran- and

Fig. 1. Effects of AGP on Carrageenan (A and B), Dextran (C and D) and Kaolin (E and F) Induced Paw Edema and Vascular Permeability

□, control (saline); ○, 45 μ M AGP; ■, 45 μ M HSA. The data are the average values of three experiments ($\pm$ S.D.). * p <0.01 as compared with control.

Fig. 2. Effects of AGP on Neutrophils Chemotactic Response to fMLP

The number of neutrophils that passed into the lower chamber through the membrane filter were measured. Concentration of AGP and HSA was $45 \mu\text{M}$. The data are the average values of three experiments ($\pm$ S.D.). * $p < 0.01$ as compared with control.

Fig. 3. Effects of AGP on Luminol Oxidation by fMLP (A) or PMA (B)

Luminol was added to neutrophils that had been incubated for 10 min with $45 \mu\text{M}$ AGP ($\circ$), $45 \mu\text{M}$ HSA ($\blacksquare$) or HBSS ($\square$), and stimulated with fMLP or PMA. The data are the average values of three experiments ($\pm$ S.D.).

kaolin-induced paw edema and vascular permeability respectively. These results suggest that AGP had anti-inflammatory effect *in vivo*.

Effects of AGP on Neutrophils Activities In an inflammatory event, neutrophils migrate, and superoxide anion are then generated. As shown in Fig. 1, AGP suppressed carrageenan-induced paw edema, which involve substantial leucocytic migration. Thus, the issue of whether AGP inhibits the chemotactic response of neutrophils to fMLP was examined by means of the Boyden-chamber method.³¹⁾ Neutrophils were placed in the upper side of the chemotaxis chamber, and fMLP, a neutrophils migration factor, was placed in the lower side. Neutrophils then pass through the pore size $3 \mu\text{m}$ filter moving to the lower side. As shown in Fig. 2, AGP decreased the number of neutrophils passing through the filter, whereas HSA had no effect. In addition, the effect of AGP on superoxide anion generation by neutrophils was examined. As shown in Fig. 3, AGP as well as HSA inhibited the generation of superoxide anion in response to fMLP and PMA.

Effects of AGP on PGE₂ Generation in Rats Since AGP suppressed both carrageenan-induced and kaolin-induced paw edema (Fig. 1), we investigated the effect of AGP on PGE₂ generation. AGP was injected into the tail vein of rats 1 h before a tail vein injection of arachidonic acid. PGE₂ generation in plasma was significantly inhibited (Table 1), whereas HSA had no effect. These results suggest that the anti-inflammatory effect of AGP in paw edema may be explained by the inhibition of the formation of PGE₂ from arachidonic acid.

Table 1. Effects of AGP on PGE₂ Generation in Rats

Arachidonic acids	Pretreatment	PGE ₂
—	—	85.95 $\pm$ 0.51
+	—	360.19 $\pm$ 16.24
+	AGP	191.01 $\pm$ 5.62*
+	HSA	369.00 $\pm$ 17.89

The data are the average values of three experiments ($\pm$ S.D.). * $p < 0.01$, significantly different from the control with arachidonic acid.

DISCUSSION

One of the consequences of acute inflammation is the leakage of plasma elements from blood vessels to the injured region and the infiltration of neutrophils. *In vivo* anti-inflammatory effects may partially depend upon AGP, but it is unclear whether AGP suppressed carrageenan-, dextran- or kaolin-induced paw edema and vascular permeability. Carrageenan, which induces paw edema and substantial leucocytic migration, is mediated by histamine and serotonin in the initial stage, while prostaglandin and bradykinin are involved in the later stages.²⁸⁾ Dextran, which induces paw edema, is mediated by histamine and serotonin, but does not involve leucocytic migration.²⁹⁾ Kaolin-induced paw edema is mediated by histamine and serotonin.³⁰⁾ As shown in Figs. 1A—F, paw edema was largest at 3, 1 and 8 h after the administration of carrageenan, dextran or kaolin, respectively. This is consistent with the characteristics described above. We also elucidated the effects of AGP on the chemotactic response of neutrophils to fMLP and on superoxide anion generation in response to fMLP and PMA. Moreover, the effect of AGP on PGE₂ generation was examined.

Leukocytes adhere to vascular endothelial cells, and then leak from blood vessels to the injured region. This process is caused by interactions between cell adhesion molecules on the leukocyte surface and on the endothelial cell surface and the migration is due to chemotactic factors.³¹⁾ The cell adhesion molecule selectin, which contains a C-type lectin domain, binds to sLe^x.³²⁾ The sLe^x form of AGP also binds to the C-type lectin domain.³³⁾ The sLe^x form of AGP is induced during inflammation.¹⁹⁾ AGP has been reported to inhibit sLe^x/E-selectin interactions.²⁰⁾ Cyclic adenosine 3',5'-monophosphate (cAMP) suppresses the activation of integrin that is the cell adhesion molecules, through the mediation of protein kinase A and RhoA.^{34,35)} AGP affects human endothelial cells *via* cAMP as a second messenger.³⁶⁾ This suggests that AGP may have an effect on the activation of integrin. Furthermore, IL-1 regulates the activation of integrin and the expression of chemokine migrating leucocytes,^{37,38)} AGP increases the secretion of an IL-1 inhibitor by murine macrophages, most likely the IL-1 receptor antagonist.^{14,15)} Accordingly, we hypothesize that neutrophils can not adhere to vascular endothelial cells and that the leakage of plasma elements from blood vessels to the injured region and the infiltration of neutrophils are suppressed, because AGP binds to selectin, thus suppressing the activation of integrin and the expression of chemokine. As shown in Fig. 2, AGP inhibited fMLP induced neutrophils migration. fMLP activates various signal transmissions mediated by G-coupled receptors, which migrate neutrophils as the result of the reconstruction of actin.³⁹⁾ However, the mechanism of action of AGP is not

well understood.

Pukhal'skii *et al.* proposed that the antioxidant activity of AGP is due to the binding of free radicals, rather than to the inhibition of neutrophils activation.⁴⁰⁾ However, fMLP might activate protein kinase C (PKC) receptors existed in neutrophils but PMA might directly activate for PKC. PKC phosphorylates and activates NADPH oxidase, resulting in the generation of superoxide anion. The activation of NADPH oxidase is transiently stimulated in the case of fMLP, and for longer periods in the case of PMA.⁴¹⁾ This tendency is consistent with results shown in Fig. 3. As shown in Fig. 3, AGP inhibits the generation of superoxide anion in response to fMLP and PMA. AGP may suppress the activation of PKC. Thus, it can be concluded that anti-oxidant activity is caused by the inhibition of signal transmission for superoxide anion generation and release after AGP stimulates neutrophils.

AGP was found to significantly suppress dextran- or kaolin-induced paw edema (Fig. 1). Sorensson *et al.* reported that AGP had a cAMP-dependent effect on human endothelial cells and inhibited the action of histamine.³⁶⁾ The findings herein suggest that AGP may affect histamine, serotonin, prostaglandin and bradykinin production *in vivo*. Moreover, AGP inhibited the generation of PGE₂ from arachidonic acid (Table 1). This suggests that AGP may have an effect on COX activity.

AGP seems to be the maintenance agent for the capillary permeability barrier.²¹⁾ This effect appears to be the result of increasing the negative charge of the capillary endothelium by sialic acid, the terminal sugar of AGP,^{22,23)} thereby reducing the transvascular transport of polyanionic macromolecules.^{21,22)} However, asialo-AGP, in which sialic acid residues are removed from AGP suppressed carrageenan-, dextran- and kaolin-induced vascular permeability. Thus, it is possible that an alternative mechanism regulates vascular permeability. The interaction between actin and myosin which is caused by activating myosin light chain kinase (MLCK) mediates the increase in intracellular calcium concentration and the activation of PKC occurs in vascular endothelial cells.⁴²⁾ As a result, the intercellular space opens, and vascular permeability is accelerated. In addition, cAMP phosphorylates MLCK, and endothelial cells are relaxed.⁴²⁾ Thus, it is possible that AGP has an effect on signal transmission mediated by PKC and cAMP and AGP suppresses vascular permeability.

AGP is an acute phase protein. We investigated whether the concentration of AGP changed as the result of the administration of carrageenan, dextran or kaolin. AGP concentrations remained constant for period of up to 12 h after the administration (data not shown). This is reasonable, because AGP concentrations begin to increase after 24 h after an acute inflammation.⁹⁾ The anti-inflammatory effects of AGP reported in the present study are independent of endogenous AGP, and dependent on the administered AGP.

In conclusion, an increase in AGP content of serum that occurs under pathological conditions may suppress surplus inflammation reactions that are induced by autacoids and neutrophils activities. It is possible that AGP may play an important role in the maintenance of homeostasis in the body. The data presented here will be useful in terms of understanding the role of acute phase proteins that are produced at

an accelerated rate during acute phase response.

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