Effects of α_1 -Acid Glycoprotein on Erythrocyte Deformability and Membrane Stabilization

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Received July 12, 2002; accepted September 24, 2002

The effects of α_1 -acid glycoprotein (AGP) on human erythrocyte membrane were examined *in vitro*. Bovine and dog AGP, in addition to human AGP or asialo human AGP were used, and the collected data were compared with that for human serum albumin (HSA). A new technique developed by Kikuchi was used to investigate erythrocyte deformability. The addition of AGPs including human AGP facilitated the passage of human erythrocytes with an average diameter of 7.2 μm suspended in phosphate buffered saline (PBS) through a 5 μm wide microchannel; hemolysis was suppressed after the passage. The stabilizing effects of AGPs on membrane were evaluated. Human AGP prevented hemolysis induced by hypotonic phosphate buffer solution. The effects of human AGP on the oxidative changes in erythrocytes exposed to oxygen radicals were investigated. Human AGP protected erythrocytes from H_2O_2 and prevented the oxidation of dihydrorhodamine 123 to rhodamine 123 from H_2O_2 . We propose that the antioxidant activity of human AGP is due to the binding of free radicals. In all studies, the effects of human AGP on erythrocytes might not be a function of the negative charge associated with sialyl residues, because the presence of *N*-acetylneuraminic acid had no effect. However, human AGP may promote microcirculation and antioxidant activity compared with HSA. No species differences in the physiological function of AGP were found. These results suggest that an increase in the AGP content of serum above the normal value found under pathological conditions facilitates the passage of erythrocytes through capillaries, stabilizes erythrocyte membranes and protects against oxidative stress, all of which are favorable for microcirculation.

Key words α_1 -acid glycoprotein; erythrocyte deformability; antioxidant activity; erythrocyte osmotic fragility

Human α_1 -acid glycoprotein (AGP) is a negatively charged acidic glycoprotein ($\text{pK}_a=2.6$; isoelectric point=2.7¹⁾), comprised of 59% protein and 41% carbohydrate²⁾; it contains 11% sialic acid.³⁾ Human AGP, a normal serum constituent (75–100 mg/dl), with a molecular weight of approximately 44100, has been reported to be an acute phase reactant with increased plasma concentration in cases of pregnancy and inflammation and in cancer and other diseases.⁴⁾ AGP has also been reported to inhibit platelet aggregation,⁵⁾ to suppress proliferative response in lymphocyte cultures induced by anti-CD3⁶⁾ and phytohemagglutinin⁷⁾ and to increase the secretion of an IL-1 inhibitor by murine macrophages, most likely the IL-1 receptor antagonist.^{8,9)} However, its biological function is not yet fully understood. Maeda *et al.* previously reported that the addition of a small amount of AGP to a human erythrocyte suspension facilitated the passage of erythrocytes through artificial membrane filters with micropores having an average diameter smaller than that of an erythrocyte, and they proposed a possible physiological role for AGP in the microcirculation.¹⁰⁾

The above findings prompted us to characterize the action of AGP on the membrane surface of erythrocytes. Thus, the present study was undertaken to investigate in detail the effects of AGP on erythrocyte membranes. Erythrocyte deformability was measured using a new technique developed by Kikuchi *et al.*¹¹⁾ We also performed an osmotic fragility test with and without AGP. The capacity of erythrocytes to resist hemolysis can be characterized as the osmotic fragility of the membrane; osmotic fragility is classically used as a general screening procedure. We also investigated the effects of AGP on the oxidative changes in erythrocytes exposed to oxygen radicals, because AGP has been reported to inhibit

neutrophil aggregation and superoxide anion generation induced by formyl-methionyl-leucyl-phenylalanine, phorbol myristate acetate¹²⁾ and phytohemagglutinin.¹³⁾ These studies were carried out using AGP and desialylated AGP to determine the role of sialic acid in this process.

MATERIALS AND METHODS

Materials Human, bovine and dog AGP (purified from Cohn fraction VI), human serum albumin (HSA), human γ -globulin, neuraminidase and dihydrorhodamine 123 (DRD) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). *N*-Acetylneuraminic acid was obtained from Nacalai Tesque (Kyoto, Japan). H_2O_2 was obtained from Santoku Chemicals Co. (Tokyo, Japan). All other materials were of reagent grade, and all solutions were prepared using deionized, distilled water.

Desialylation of AGP Human AGP was desialylated enzymatically, using methodology described by Primožic and McNamara,¹⁴⁾ 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 was 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.¹⁵⁾ 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.

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Preparation of Erythrocyte Suspension Venous blood from the anticubital veins of healthy volunteers was collected in disposable syringes with a 21-gauge needle. After centrifugation at 3000 rpm for 10 min, the plasma and buffy coat were removed and replaced with phosphate buffered saline (PBS) (pH 7.4), and subsequently washed two times with PBS by repeated resuspension and centrifugation at 1000 rpm for 10 min. A 50% erythrocyte suspension was prepared in PBS.

Erythrocyte Deformability AGP, dissolved in PBS, was added to the washed erythrocytes, which were incubated for 3 h at 37 °C. Erythrocyte deformability was determined by Kikuchi's microchannel method.¹¹⁾ The time required for 100 μ l of erythrocytes to pass through a microchannel (MC-FAN; Hitachi Haramachi Electronics Co., Ltd.; width, 5 μ m; length, 30 μ m) was determined at a pressure of 20 cm H₂O. After filtration the erythrocytes were centrifuged at 3000 rpm for 3 min, and spectrophotometric measurements were then made on the supernatant at 540 nm to quantify the degree of hemolysis.

Osmotic Fragility Test Erythrocyte osmotic fragility was measured using washed erythrocytes, after 30 min incubation of the erythrocytes at 37 °C in solutions of 1.0—8.0 g/l phosphate buffered NaCl with and without AGP. The erythrocytes were centrifuged at 3000 rpm for 3 min, and spectrophotometric measurement of the supernatant was then made at 540 nm to quantify the degree of hemolysis.

Effects of AGP on Exposure of Erythrocytes to Oxygen Radicals A stock solution of 25 mM H₂O₂ was added to washed erythrocytes with and without AGP, and the resulting suspension incubated at 37 °C for 3 h in a shaking water bath. The erythrocytes were centrifuged at 3000 rpm for 3 min and the absorption of the supernatant at 540 nm was used to quantify the degree of hemolysis.

Reactive Oxygen Species (ROS) Quenching Capacity H₂O₂ oxidizes DRD to rhodamine 123 (RD), which fluoresces at 536 nm when excited at 500 nm. Two milliliters samples of AGP (45 μ M) and DRD (5 μ M) were prepared in 67 mM phosphate buffer, and at a predetermined time H₂O₂ (25 mM) was added. The progress of the reactions was followed spectrophotometrically by a fluorescence measurement of the RD formed. Controls were performed without any additives. Quenching of control was 0%.

Statistics Where possible, statistical analyses were performed by the Student's *t*-test.

RESULTS

Effects of AGP on Erythrocyte Passage through Microchannel Effects of bovine and dog AGP in addition to human AGP, HSA and *N*-acetylneuraminic acid on erythrocyte deformability are shown in Fig. 1. When the concentration of human AGP added to erythrocyte suspensions was increased, the passage time of erythrocytes through microchannels with an average diameter of 5 μ m decreased. The effects of bovine, dog and asialo-human AGPs on the passage times were similar to those for human AGP; however, *N*-acetylneuraminic acid had no effect. This suggests that the enhancement was not a function of a negative charge related to the sialyl residues. The passage times for other serum proteins, HSA and γ -globulin were slightly but significantly slower

Fig. 1. Effects of AGPs on the Time Required for 100 μ l Erythrocytes to Pass through Microchannels

11 μ M and 23 μ M AGP (normal concentration level). 45 μ M AGP (above normal concentration level). A, without serum protein; B, 11 μ M human AGP; C, 23 μ M human AGP; D, 45 μ M human AGP; E, 45 μ M human asialo-AGP; F, 900 μ M *N*-acetylneuraminic acid; G, 45 μ M dog AGP; H, 45 μ M bovine AGP; I, 45 μ M HSA; J, 45 μ M γ -globulin. The data are average values of 3—4 experiments ($\pm$ S.D.). * p < 0.005 as compared with HSA. ψ p < 0.01 as compared with HSA.

Fig. 2. Effects of AGPs on the Hemolysis of Erythrocytes during Passage through Microchannels

D, E, F, G, H and I are the same as shown in Fig. 1. The data are average values of 3—4 experiments ($\pm$ S.D.). * p < 0.01 as compared with HSA.

than those for AGPs, whereas the degree of hemolysis after passage was suppressed by nearly the same amount by AGPs and HSA, except for *N*-acetylneuraminic acid (Fig. 2).

Effects of AGP on Osmotic Fragility of the Erythrocyte Membrane To determine whether AGP contributes to the membrane stability or not, we performed an osmotic fragility test. Human AGP suppressed hemolysis induced by hypotonic phosphate buffer solution, and significant suppression was observed at 74 and 89 mOsm (data not shown). The suppressive effects of different AGPs, HSA and *N*-acetylneuraminic acid on hemolysis were determined at 74 and 89 mOsm. Effects of HSA, bovine, dog and asialo-human AGPs on hemolysis were similar to those of human AGP; however, *N*-acetylneuraminic acid had no effect (Table 1).

Antioxidant Activity of AGP to Erythrocytes Erythrocytes exposed to 25 mM H₂O₂ begin to undergo hemolysis after 1 h, and this increased gradually up to 3 h. The addition of different AGPs suppressed the hemolysis (Fig. 3). Table 2 shows the quenching capacities of human AGP and asialo-human AGP on DRD oxidation by H₂O₂. Both AGPs abolished the oxidation by H₂O₂ significantly. Interestingly, the antioxidant activities observed for AGPs were significantly higher than those of HSA (Fig. 3, Table 2).

Table 1. Effects of AGPs on Hemolysis at 74 mOsm

	Hemolysis (% of control)
23 μ M Human AGP	84.4 $\pm$ 1.7
45 μ M Human AGP	82.6 $\pm$ 2.9
45 μ M Human asialo-AGP	82.3 $\pm$ 3.1
900 μ M <i>N</i> -Acetylneuraminic acid	104.5 $\pm$ 4.3*
45 μ M Dog AGP	84.0 $\pm$ 1.5
45 μ M Bovine AGP	82.6 $\pm$ 2.5
45 μ M HSA	82.8 $\pm$ 3.3

The data are average values of 3–4 experiments ($\pm$ S.D.). * p <0.01 as compared with HSA.

Table 2. Quenching of H₂O₂ Oxidation of DRD by Human AGP and HSA

	Quenching (%)
45 μ M Human AGP	96.6 $\pm$ 4.2*
45 μ M Human asialo-AGP	92.7 $\pm$ 3.8*
900 μ M <i>N</i> -Acetylneuraminic acid	0.5 $\pm$ 5.3 [#]
45 μ M HSA	70.2 $\pm$ 11.7

The data are average values of three experiments ($\pm$ S.D.). * p <0.01 as compared with HSA. [#] p <0.001 as compared with HSA.

Fig. 3. Effects of AGPs on Hemolysis of Erythrocytes Induced by H₂O₂

D, E, F, G, H and I are the same as shown in Fig. 1. The data are average values of 3–4 experiments ($\pm$ S.D.). * p <0.01 as compared with HSA. [#] p <0.001 as compared with HSA.

DISCUSSION

Our results show that the passage of human erythrocytes suspended in PBS through a microchannel 5 μ m wide was facilitated and that hemolysis was suppressed by various AGPs (Figs. 1, 2). Although the reason AGP exhibited significant effects among the three serum proteins tested is not known, the effects for AGP might be due to the structural properties with five carbohydrate chains, different from HSA and γ -globulin. However, it should be noted that *N*-acetylneuraminic acid, a component of AGP, has no effect on the membrane. We know the effects on passage and hemolysis are not a function of negative charge on the sialyl residues (Figs. 1, 2). The issue of how AGP affects erythrocyte deformability is not fully understood. Maeda *et al.* previously showed that human AGP is bound to the surface of the erythrocytes as evidenced by fluorescent antibody staining.¹⁰ They suggest that human AGP added to the suspension forms a lubricating film on the surface of erythrocytes by binding to the surface, thus reducing the friction between the erythro-

cytes and the microchannel.

They also showed that human AGP is able to stabilize the membrane by binding to the surface of the erythrocytes. In fact, human AGP prevented hemolysis induced by hypotonic phosphate buffer solution (Table 1), but the sialic acid may not play a role in this effect (Table 1).

The present studies show that human AGP, at physiological concentrations, protects erythrocytes from H₂O₂ (Fig. 3). Pukhal'skii *et al.* reported that human AGP inhibits neutrophil superoxide anion generation and suggested that the antioxidant activity of AGP is due to the binding of free radicals, rather than to the inhibition of neutrophil activation.¹³ The data obtained here support this proposal. Based on this mechanism, it is possible that AGP prevents the oxidation of DRD to RD from H₂O₂ (Table 2).

It has been reported that bovine AGP inhibits ischemia/reperfusion injury by preventing apoptosis and inflammation¹⁶ and prevents Gram-negative infections.¹⁷ Since AGP is highly homologous among the species,^{18,19} it seems reasonable that there are species differences in the physiological function of AGP which have not been found (Figs. 1–3, Table 1).

We propose that an increase in the AGP content of the serum above the normal value found under pathological conditions⁴ facilitates the passage of erythrocytes through capillaries, stabilizes erythrocyte membranes and protects against oxidative stress, which would be a favorable situation for microcirculation. In fact, under pathological conditions of highly elevated fibrinolytic or clotting activity, antigen–antibody complex formation and oxidative stress takes place which impedes blood circulation in the capillaries. However, an elevation in AGP levels in the plasma should facilitate the microvascular passage of erythrocytes and stabilize the membrane. These limited data indicate that AGP may function as an important factor in homeostatic mechanisms that operate during microcirculation, although a molecular mechanism for the function of AGP remains to be elucidated.

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