FULL PAPER *Physiology*

Akita dogs possess GLUT1 in erythrocytes, and Na,K-ATPase activity enables more efficient ascorbic acid recycling

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ABSTRACT. We investigated hematologic characteristics of healthy Akita dogs. All were found to contain glucose transporters, GLUT1 and GLUT4, in erythrocyte membrane, whereas Beagle and any other Western dogs have only GLUT4. Of 47 Akitas, ten showed high K and low Na concentrations with elevated glutathione (GSH) in erythrocytes due to Na,K-ATPase activity in the membrane (HK). Akitas showed increased capacity for recycling vitamin C or ascorbic acid (AA) from oxidized ascorbic acid (DHA) compared to Beagle dogs. Particularly, HK Akitas performed even greater AA recycling and ferricyanide reduction than normal Akitas which have normal GSH, low K and high Na concentrations (LK). All HK Akitas also had stomatin in erythrocyte membrane, while half of LK Akitas had it at lower levels than HK Akitas. Stomatin did not have any influence on AA recycling. GLUT1, Na,K-ATPase and stomatin in erythrocytes are characteristics of Akita dogs, and the high prevalence of these proteins suggests their positive roles in biological efficiency.
KEY WORDS: Akita dog, ascorbic acid, erythrocyte, GLUT1, Na,K-ATPase

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GLUT1 is a facilitative glucose transporter mediating entry of D-glucose and oxidized ascorbic acid (DHA) across plasma membranes [25]. DHA once taken into the cell is immediately reduced to ascorbic acid (AA) by GSH or NADPH, allowing efficient AA recycling [16, 17]. All newborn animals contain high levels of GLUT1 in fetal erythrocytes [11, 19, 27]. In most animals, GLUT1 is rapidly lost during the neonatal period in which fetal erythrocytes are replaced by adult cells [10]. However, human, higher primates and a few other species retain high levels of GLUT1 throughout life [18]. Stomatin functions to enhance GLUT1-mediated DHA uptake and concomitantly reduce glucose transport. It is considered as a compensation mechanism in animals unable to synthesize vitamin C (AA), protecting cells from oxidative stress [26], and plays essential roles in the biosynthesis of collagen, carnitine and neurotransmitters [23].

Dogs can produce vitamin C (AA) and do not possess GLUT1 in erythrocytes [4, 19]. We have recently discovered that they have GLUT4 instead, and about half of Japanese Shiba dogs possess GLUT1 besides GLUT4 [20–22]. Shiba dogs with GLUT1 show much faster uptake of glucose and DHA and greater capacity for recycling AA than those with only GLUT4. Expression of GLUT1 in erythrocytes is inherited in an autosomal recessive mode [22]. Stomatin is found in some of Shiba dogs, however, it is not associated with expression or function of GLUT1 [22].

There is another breed Akita in Japan. Both Shiba and Akita dogs are known for their high K and low Na concentrations (HK) in erythrocytes due to the high Na,K-ATPase activity in the membrane [6], while normal dogs have low K and high Na concentrations (LK) because they lose the enzyme during reticulocyte maturation [12]. HK dog erythrocytes contain high GSH concentrations due to increased glutamate uptake by Na gradient across the membrane created by the enzyme [13]. The trait is inherited in an autosomal recessive mode [14]. The HK phenotype is found all over Japan, and its prevalence is 26% in Akitas and 37% in Shibas [6]. Both breeds are genetically close to each other. Their high prevalence of HK led us to wonder if Akitas also possess GLUT1 and stomatin in erythrocytes.

Vitamin C and GSH work together creating an efficient reductive system protecting cells against oxidative stress [16, 17]. Thus, in the present report, we surveyed GLUT1 and HK phenotypes in Akita dogs and studied AA recycling and other hematologic characteristics in this breed.

MATERIALS AND METHODS

A total of 47 purebred Akita dogs (age 1–13 years) from Tokyo, Kanagawa and Saitama areas were used. All dogs were privately owned and fed dry dog food not containing vitamin C. Their blood relation is not known, except for two dogs (aunt and niece). After informed consent, venous blood was taken from the dogs fasted overnight and collected into tubes containing EDTA-2Na or heparin. Six Beagle dogs were also used as controls. All dogs were subjected to biochemical tests including total protein (TP), albumin (Alb),

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alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkali phophatase (ALP), total cholesterol (TCh), triglyceride (TG), total bilirubin (Tbil), creatinine (Cre), blood urea nitrogen (BUN), glucose, inorganic phosphate (IP), Ca, Na, K and Cl, and hematological tests to confirm their state of health. Plasma AA, erythrocyte Na, K and GSH concentrations, and AA recycling capacity were determined as described previously [21, 22]. Osmotic fragility test was performed by the Parpart method [24].

AA recycling capacity and AA-dependent ferricyanide reduction: Erythrocytes were separated from blood by centrifugation, washed three times with 0.9% NaCl and resuspended at 10% hematcrit in the medium containing 137 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5 mM glucose and 20 mM sodium hydrogen phosphate buffer (pH 7.4). Erythrocytes were incubated with 1, 5 and 10 mM DHA at 37°C for 20 min, and washed three times with 0.9% NaCl. Intracellular AA was assayed as described previously [21]. For measuring the reduction of extracellular oxidant, aliquots of AA-loaded erythrocytes were incubated with 10 vol. of the medium containing 1 mM K_3FeCN_6 for 30 min at 37°C. The amount of ferrocyanide generated was determined according to the method of Avron and Shavit [2] using 1,10-phenanthroline as an indicator.

Immunoblot analyses: Preparation of erythrocyte membrane ghosts and immunoblot analyses were conducted for GLUT1, GLUT4, actin and stomatin as mentioned previously [20, 22]. However, Clarity Western ECL substrate (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and Amersham Hyperfilm TM ECL (GE Healthcare, Buckinghamshire, U.K.) were used for detection of membrane proteins. All experimental procedures met with the approval of the Laboratory Animal Ethics Committee, Azabu University.

Statistics: Values are expressed as the mean ± SD of dogs used in the experiments. Any difference between groups was tested by Welch's *t*-test. A *P* value less than 0.05 is considered significant.

RESULTS

All Akita dogs had GLUT1 in erythrocyte membranes (Fig. 1). Each dog showed a typical broad band at MW 47,000–54,000, the intensity of which was almost similar to that obtained from a Shiba dog. GLUT4 was also uniformly present in all Akitas as observed in Shiba and Beagle dogs in Fig.1 and previous reports [20–22].

Of 47 Akitas, ten (21%) contained high K and low Na concentrations with an elevated GSH in erythrocytes (HK cells), whereas 37 other Akitas had low K, high Na and normal GSH concentrations (LK cells), as shown in Table 1. Ascorbic acid recycling in erythrocytes of these dogs resulted in AA accumulation only in its reduced form. HK Akita dogs regenerated more ascorbic acid from DHA than LK Akitas (Fig. 2). The level of AA recycled from DHA in LK Akitas was similar to that observed in LK Shiba dogs with GLUT1 in the previous report [21]. Beagle dogs bearing only GLUT4 in erythrocytes showed less efficient AA recycling than LK Akitas. Ferricyanide reduction by erythrocytes loaded with AA increased almost linearly with intracellular AA concentration (Fig. 3). HK Akitas which accumulated higher AA concentrations were able to reduce more ferricyanide than LK Akitas.

Stomatin was detected by immunoblotting at MW 30,000 (Fig. 4). All HK Akitas presented stomatin in erythrocytes, while half of LK Akitas expressed it at lower levels and the rest showed hardly anything. All HK and some of LK Shiba

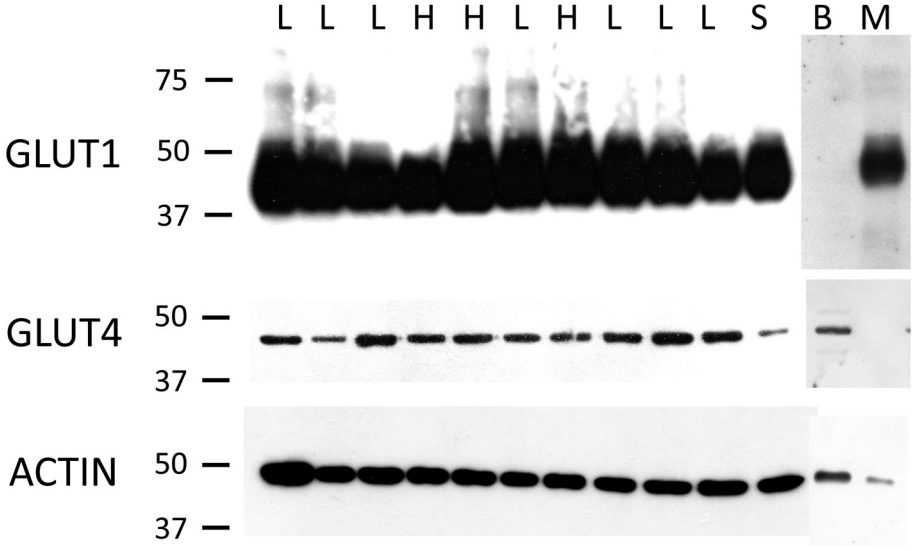

Fig. 1. Immunoblots of erythrocyte GLUT1, GLUT4 and actin from HK and LK Akita dogs. H, HK Akita; L, LK Akita, S, LK Shiba with GLUT1; B, Beagle; M, Human. Each protein loading of the SDS-PAGE gel was 5 μg/lane, except for 0.5 μg/lane for human GLUT1 and actin.

Table 1. Na, K and GSH concentrations in HK and LK Akita dogs

	HK Akitas (n=10)	LK Akitas (n=37)	Beagles (n=6)
Plasma Na (mmol/L)	144.9 ± 5.6	142.5 ± 5.1	138.8 ± 3.1
Plasma K (mmol/L)	4.4 ± 0.4	4.1 ± 0.2	4.6 ± 0.3
RBC Na (mmol/L RBC)	12.4 ± 1.4**	109.7 ± 8.2	95.4 ± 9.3
RBC K (mmol/L RBC)	106.7 ± 11.8**	5.3 ± 1.8	6.2 ± 1.6
RBC GSH (μmol/g Hb)	38.0 ± 10.8**	10.1 ± 1.3	9.5 ± 0.7

Data are expressed as the mean ± SD. Asterisks indicate significant differences between HK and LK Akitas: **P<0.01. RBC, red blood cell or erythrocyte; GSH, glutathione.

dogs showed similar levels of stomatin as reported previously [22]. There was no significant difference in AA recycling capacity between LK Akitas with and without stomatin (data not shown).

Hematologic examination showed lower RBC counts, Hb and MCHC values in HK Akitas than LK Akitas or Beagle dogs (Table 2). On the contrary, MCV was larger in HK Akitas (66.5 ± 2.5 fL) than LK Akitas (59.9 ± 4.6 fL), although it was within the range of Beagle dogs (69.4 ± 2.7 fL). Osmotic fragility curves of LK Akitas and Beagles were almost similar, providing 50% lysis at saline concentrations of 0.41 ± 0.03% and 0.40 ± 0.02%, respectively. In HK Akitas, hemolysis started to occur at 0.6 to 0.8% saline concentration and provided 50% lysis at 0.46 ± 0.03% saline concentration, indicating increased osmotic fragility (Fig. 5).

DISCUSSION

All Akita dogs had GLUT1 and GLUT4 in erythrocytes. They recycled AA from DHA more efficiently than Beagle dogs with only GLUT4. Akitas with high GSH concentration due to Na,K-ATPase activity showed even greater AA recycling capacity and ferricyanide reduction than normal LK Akitas.

Entrance of DHA is a rate-limiting step of AA recycling. When DHA enters the cells, it is immediately reduced to AA primarily by GSH-dependent DHA reductase with a small contribution from NADPH-dependent DHA reductases [16,

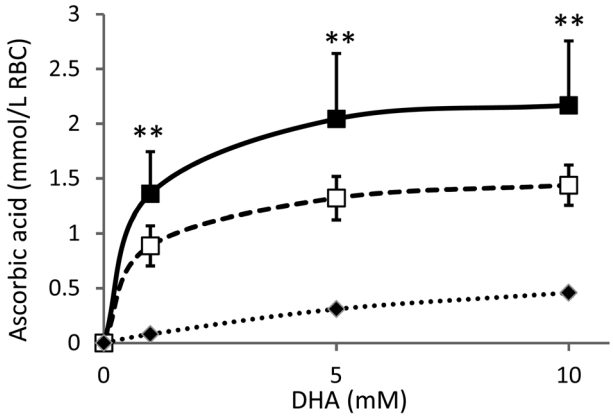

Fig. 2. Ascorbic acid recycling capacity in erythrocytes of HK and LK Akita dogs. Intracellular AA was measured after 20-min incubation with DHA. Data are expressed as the mean ± SD of HK Akitas (■, n=10), LK Akitas (□, n=37) and Beagles (◆, n=6). Asterisks indicate significant differences between HK and LK Akitas: **P<0.01.

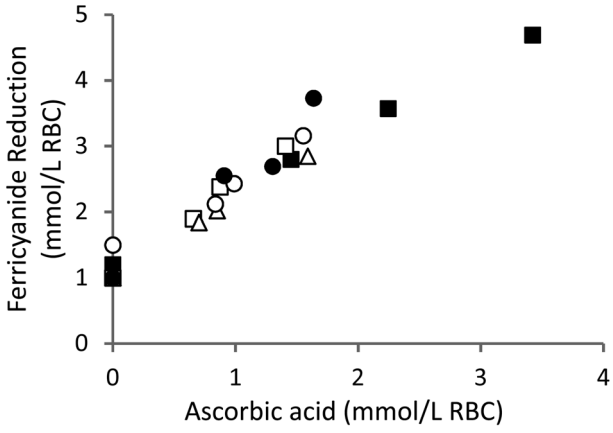

Fig. 3. Plot of ferricyanide reduction and ascorbic acid concentration in erythrocytes from two HK (●, ■) and three LK (○, □, △) Akita dogs. Each symbol represents data obtained from the same animal.

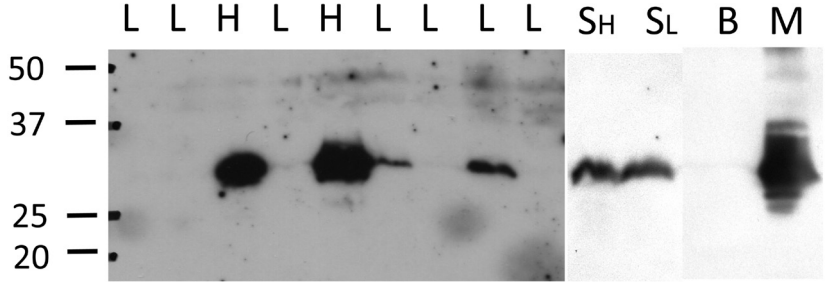

Fig. 4. Immunoblots of erythrocyte stomatin from HK and LK Akita dogs. H, HK Akita; L, LK Akita; SH, HK Shiba; SL, LK Shiba; B, Beagle; M, Human. Each protein loading of the SDS-PAGE gel was 100 μg/lane, except for 5 μg/lane for human.

Table 2. Hematological parameters of HK and LK Akita dogs

	HK Akitas (n=10)	LK Akitas (n=37)	Beagles (n=6)
RBC (10^4/mm^3)	634 ± 76**	771 ± 91	790 ± 36
PCV (%)	42.7 ± 5.0	46.0 ± 5.4	54.8 ± 2.4
Hb (g/dL)	12.1 ± 1.9**	15.0 ± 1.8	17.1 ± 0.7
MCV (fL)	66.5 ± 2.3**	59.7 ± 4.5	69.4 ± 2.7
MCH (*p*g)	19.0 ± 1.3	19.4 ± 1.6	21.7 ± 0.7
MCHC (g/dL)	28.6 ± 2.4**	32.6 ± 1.7	31.3 ± 0.2

Data are expressed as the mean ± SD. Asterisks indicate significant differences between HK and LK Akitas: ** P<0.01. RBC, red blood cell counts; PCV, packed cell volume; Hb, hemoglobin concentration; MCV, mean corpuscular cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

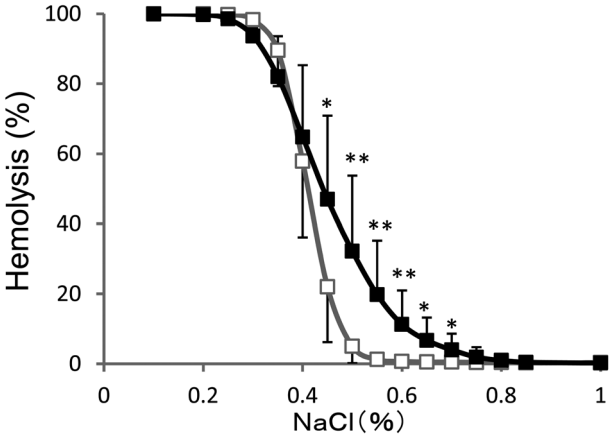

Fig. 5. Osmotic fragility curves for the erythrocytes from HK and LK Akita dogs. Data are expressed as the mean ± SD of HK (■, n=10) and LK (□, n=20) Akita dogs. Asterisks indicate significant differences between HK and LK Akitas: * P<0.05, ** P<0.01.

17]. GSH is converted to oxidized glutathione (GSSG), but readily reduced by the NADPH-dependent glutathione reductase system [1]. Thus, high GSH concentration likely enables more efficient DHA reduction. AA recycled from DHA is trapped within cells, accumulated against the concentration gradient and slowly leaves cells by diffusion. Ferricyanide, a potent oxidizing agent unable to enter cells, is reduced to ferrocyanide by intracellular AA which provides electrons through the membrane [7, 15, 17]. Extracellular free radical derived from oxidation of AA can be reduced back to AA by this mechanism without crossing the cell membrane. Even extracellular DHA can be reduced at the rate depending on intracellular AA concentrations [8]. In this way, Akita dogs containing GLUT1 have greater antioxidant protection than normal dogs, and some HK Akitas with hereditary high GSH concentration thus obtain extra protection.

However, high GSH and K concentrations do not always benefit dog erythrocytes. As observed in Akitas in Fig. 5, marked increase in osmotic fragility was reported in HK dogs compared to LK dogs [3, 5]. Pseudokalemia occurs during the storage of blood taken from HK Akitas, and for this reason, they cannot be used as blood donors [5]. Moreover, dogs with high GSH suffer more severe hemolytic anemia after onion ingestion, because GSH accelerates the oxidative stress produced by n-propylthiosulfate in onion [28]. Nevertheless, the HK gene has survived for more than 2,000 years, and HK phenotype is frequently found in Japanese breeds to date.

Besides GLUT1 and Na,K-ATPase, stomatin was found in erythrocyte membrane of Akita and Shiba dogs. According to Komatsu *et al.*, all these proteins are present in reticulocytes of Beagle dogs at high levels, but extruded into exosomes during cell maturation and completely lost from erythrocytes [9]. Meanwhile, some Japanese mongrel dogs retain both Na,K-ATPase and stomatin in mature red cells. A functional association between stomatin and Na,K-ATPase activity in dog erythrocytes has been suggested, and the level of stomatin can be a genotypic marker for the HK/LK phenotypes. In our work, all HK Akitas had stomatin, and half of LK Akitas had it at lower levels. Stomatin in human RBCs functions to enhance GLUT1-mediated DHA uptake [18]. However, LK Akitas with or without stomatin showed similar AA recycling capacity, as LK Shiba dogs did in the previous report [22]. Thus, stomatin does not likely influence GLUT1-mediated DHA uptake in dog erythrocytes.

Why all Akitas possess GLUT1 in erythrocytes remains an intriguing question. According to Fujise *et al.*, Korean dogs have Na,K-ATPase in mature erythrocytes as well as Japanese dogs, suggesting that gene mutation for HK occurred in Korean dogs in ancient times and later spread across Japan [6]. Although GLUT1 has not been determined in Korean dogs to date, its discovery in both Akita and Shiba breeds in our study would suggest that the gene for GLUT1 was introduced in ancient Japan before Japanese breeds were established. Until the late 19th century, Akita dog had been a medium-sized hunting dog in northwest Japan. When dogfighting boomed, Akita grew larger by mating with larger western breeds, resulting in considerable changes in its appearance and temperament. Later, careful breeding was served to reconstitute the strain. The present Akita is no longer a hunting or fighting dog, but a gentle family dog with a large body frame. Based on the history of Japanese dogs, we presume that the recessive allele for GLUT1 in Akitas became homozygous during recent breeding and only offspring with GLUT1 survived.

Overall, expression of GLUT1, Na,K-ATPase and stomatin is characteristics of Akita dogs. GLUT1 and Na,K-ATPase are not genetically linked, however, their combination likely provides stronger antioxidant protection, contributing to their health. The function of stomatin in dog erythrocytes is not known. A further study is needed to understand the detailed mechanisms of these membrane proteins expressed in mature erythrocytes of Akita dogs.

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