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Prolonged Bleeding Time in Aleutian Mink Associated with a Cyclo-Oxygenase-Independent Aggregation Defect and Nucleotide Deficit in Blood Platelets

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SUMMARY

A prolonged mean template bleeding time of 13 minutes was present in nine Aleutian mink affected with Chediak-Higashi syndrome (CHS) compared with 4 minutes in dark control mink. The concentrations of blood platelets in normal and affected animals did not differ significantly. However, in mink with CHS, a marked disturbance of platelet response to collagen was present. Administration of aspirin and indomethacin completely blocked CH platelet response to collagen. Blood platelet adenosine triphosphate and adenosine diphosphate values from mink with CHS were significantly less than those of normal mink, and the platelet adenosine triphosphate/adenosine diphosphate ratios were 10.31 in affected mink and 2.74 in normal mink. These findings are consistent with our previous investigations in affected cattle and persons and indicate that a "storage pool disease" of platelets exist in the mink with CHS.

It has been commonly observed that Aleutian mink have a bleeding tendency under ranch conditions (Fig 1). Aleutian mink are known to have many characteristics common to other species with Chediak-Higashi syndrome (CHS) and are considered to have a form of CHS. The CH syndrome is a genetic disorder in persons,[1] mink,[2] cattle,[3] mice,[4] cats,[5] and a killer whale,[6] with an autosomal recessive mode of inheritance. In CHS, there is hypopigmentation due to abnormal melanosome formation, recurrent pyogenic infections, enhanced susceptibility to all viral infections such as Aleutian disease, enlarged lysosomes recognizable in secretory cells, and a hemorrhagic diathesis.[7] A study[8] of the coagulation and fibrinolytic systems in mink failed to disclose the cause for the hemorrhagic tendency. Mink affected with CHS were reported to have had normal blood platelet counts. Whole blood serotonin, and therefore presumably platelet serotonin, of CHS-affected mink[9,a] are greatly reduced,[10] as in persons,[11] cattle, and mice.

The observation of an Aleutian mink hemorrhagic tendency in association with normal soluble clotting factors and normal platelet counts, but low concentrations of blood serotonin, indicated that a platelet storage pool disorder (SPD) may exist, such as we have demonstrated in other animals with CHS.[12] This report characterizes the platelet abnormality by quantitating several biochemical and functional features of platelets from CHS-affected and normal mink. We have investigated the effect of two cyclo-oxygenase inhibitors on platelet function and have shown that dysfunction in platelets from affected mink is compounded by these agents.

Materials and Methods

Animals—The mink used were adult males and females obtained from commercial breeders and maintained by Washington State University and Michigan State University.[7] Because Aleutian disease (AD) influences platelet counts and fibrinogen levels,[13] the mink used in the study were found to be free of AD before the start of the experiments, as determined by the absence of antibody to AD virus as measured by counter electrophoresis.[14] Blood smears were examined prior to experiments in order to identify CHS-affected mink by the presence of giant granules in leukocytes.[1]

Bleeding Time Determination—Bleeding time determinations were conducted on nine dark and nine CHS-affected mink, according to established methods.[15,16] In the mink, triplicate skin incisions were made on the proximal part of the thoracic limb or ventral aspect of the neck.

Blood and Platelet Collection—Blood samples were collected from the jugular vein (chemical analyses) or directly from the heart (aggregation test) of mink while under ether anesthesia.

Two methods of blood collection were used, one for nucleotides on 6 dark and 6 CHS-affected mink and another for aggregations on 20 dark and 20 CHS-affected mink. In the first, anticoagulation was achieved with 3.8% trisodium citrate solution (pH 7.4); the citrate solution was mixed with blood (1:10, v/v). Platelet-rich plasma (PRP) was obtained by 180 × g centrifugation for 10 minutes (with up to an additional 10 minutes at 180 × g if required). Samples were removed for counts, and the remainder were stored in 3-ml plastic tubes for processing in nucleotide assays. For

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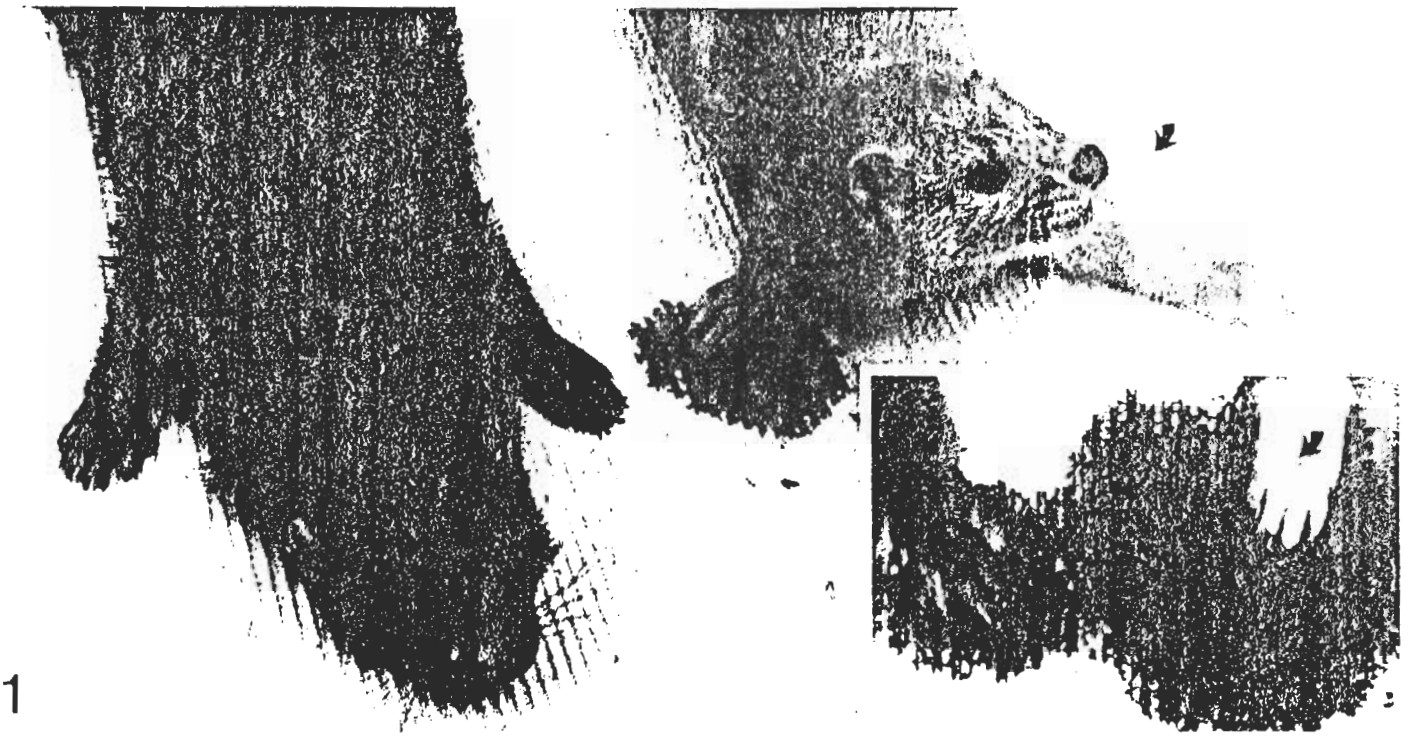

Fig 1—The amount of bleeding due to toenail clipping, a common bleeding technique for mink, in an Aleutian Chediak-Higashi (CH) mink (arrow) compared with the normal genotype after 1.5 minutes and after 8 minutes (insert).

aggregometry, the blood sample was drawn through a 21-gauge, 1-½ inch needle into a 10-ml syringe containing 3.8% trisodium citrate solution (0.8 ml of citrate to 9.20 ml of blood). The blood mixture was transferred to 15-ml plastic centrifuge tubes and centrifuged for 10 minutes at 200 × g to obtain PRP. The specimens used for platelet aggregations (0.5-ml portions of PRP) were transferred to siliconized glass tubes, and the remaining specimen was centrifuged at 1,200 × g for platelet-poor plasma (PPP).

From 1.5 to 2.0 ml of PRP was obtained from a 10-ml blood sample. The PRP assayed for nucleotides, within 15 minutes after centrifugation, was mixed with K$_3$EDTA-ethanol (1:1, v/v) solution in the same tube. The samples were held at 0 to 4 C, with mixing each minute for 5 minutes and not longer than 10 minutes. A supernate was then obtained, according to the method of Holmsen et al,[17] and samples were stored for 2 months or less at −60 C. Platelet counts of PRP, PPP, and whole blood from the same sample were performed in RBC chambers with phase microscopy. The PRP for aggregations was capped after collection and held at room temperature for 30 minutes before performing platelet aggregations.

Platelet Aggregations—Platelet aggregations were done on a Payton dual channel aggregometer, equipped with a dual-pen recorder.[b] Twenty blood samples from normal and 20 blood samples from CHS-affected mink were analyzed in pairs. While in the aggregometer, the PRP was continuously stirred at 900 rpm and maintained at 37 C. The PPP was used to set 100% and PRP 0% transmittance. Aggregation was induced by 20-µl additions of collagen (0.03 mg of collagen (bovine)/ml, Bio-Rad protein assay)[c] and ADP (5 µ*M*, Sigma). For 2 minutes before and 5 minutes after addition of the aggregating agent, the sample was held in the aggregometer. Collagen and ADP were prepared according to Holmsen et al.[18] Approximately 1-ml portions were pipetted into small vials and frozen for later use. When needed for platelet aggregation, a vial was thawed, vigorously mixed, and kept in an ice bath during use. In trials testing cyclo-oxygenase inhibitors, 7.7 mg of aspirin[a] or 0.55 mg of indomethacin[a] in 3 ml of water were injected intraperitoneally 1 hour ± 30 minutes before blood sample collection. The blood concentrations were not determined, and the amounts injected simply reflected inhibiting doses extrapolated from doses reported for other species.[19,20]

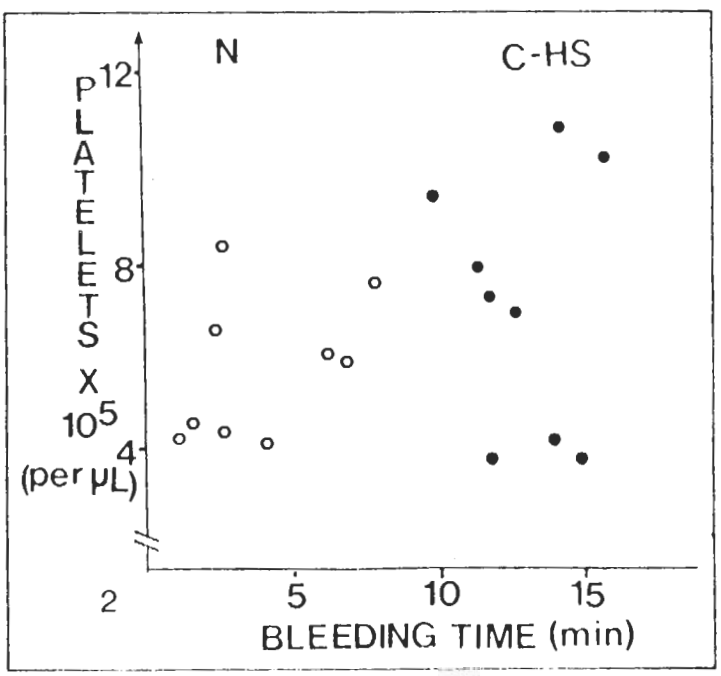

Fig 2—Platelet counts and bleeding times from normal mink (N, ○) and mink affected with CH syndrome (CHS; ●); triplicate determinations.

Aggregation rate was determined by locating the steepest portion of the aggregation curve within 5 minutes of addition of the aggregating agent and calculating the slope in arbitrary chart units of change of optical density per minutes.

Nucleotides—The determination of nucleotide content of ethanol-extracted platelets (20 µl) from PRP was performed, as described by Holmsen et al,[17,21] with the exception that the firefly

[a] Houston Instruments, Bossalow, NY.
[b] Bio-Rad Laboratories, Richmond, Calif.

lantern extract (Sigma) was subjected to homogenization for 15 to 30 s in a glass TenBroeck homogenizer.

Statistical Analysis—Results were analyzed by means of Student's *t* test (unpaired) and unless otherwise stated, expressed as means ± SD. Differences with $P < 0.01$ were considered to be significant.

Results

The results of bleeding times of 18 mink are illustrated (Fig 2). Nine mink with CHS had a mean bleeding time of 12.9 ± 1.4 minutes, whereas the nine control mink had a bleeding time of 4.5 ± 1.0 minutes ($P < 0.01$).

In Table 1, the analysis of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from PRP of nine affected CHS and nine control mink is presented. The concentrations of platelet adenine nucleotides of affected animals were 28.5% of the ATP and 25.1% of the ADP in control platelets (significantly different at $P < 0.01$). The ATP/ADP ratio was 2.74 in control mink platelets compared with 10.31 in the CHS-affected mink platelets.

The data from platelet aggregation studies are presented (Fig 3, 4, and 5; Table 2). The platelet counts were similar

TABLE 1—Platelet ATP and ADP from Mink Affected with Chediak-Higashi Syndrome

Mink No.	ATP (μmoles/10^{11} cells)	ADP (μmoles/10^{11} cells)	ATP/ADP (means of ratios)
Normal mink (n = 6)			
1	3.00*	2.09	1.44
2	10.07	1.72	5.86
3	11.00	4.29	2.26
4	10.58	4.70	2.25
5	10.18	4.40	2.32
6	9.83	4.29	2.28
Means (± SD)	9.11 (3.02)	3.58 (1.31)	2.74
Affected mink (n = 6)			
7	2.88	0.07	40.55
8	1.87	0.76	2.44
9	1.90	2.77	0.69
10	3.95	0.82	3.70
11	2.10	0.89	2.37
12	2.87	0.07	12.10
Means (± SD)	2.60 (0.81)	0.90 (0.99)	10.31

* Each tabular entry represents the mean of triplicate assay from each mink. ATP = Adenosine 5'-triphosphate; ADP = adenosine 5'-diphosphate.

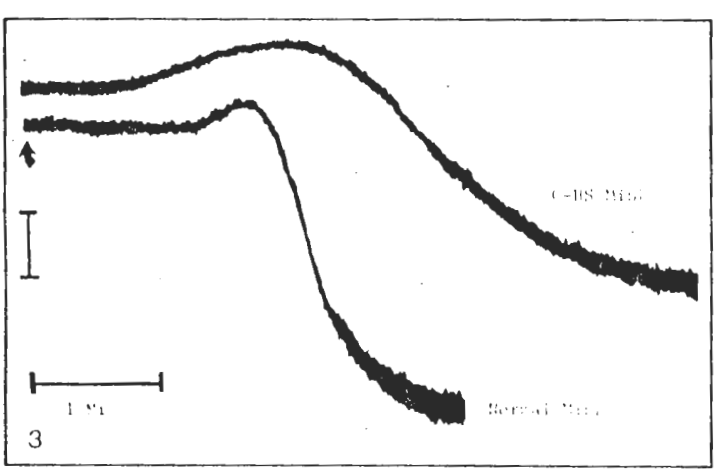

Fig 3—Aggregation curves in CHS-affected mink and normal mink, induced with collagen (addition at arrow).

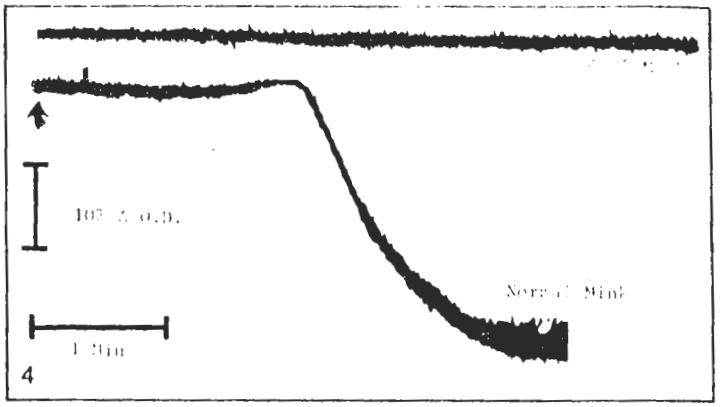

Fig 4—Aggregation curves in CHS-affected mink and normal mink, induced with collagen after in vivo treatment with aspirin (addition at arrow).

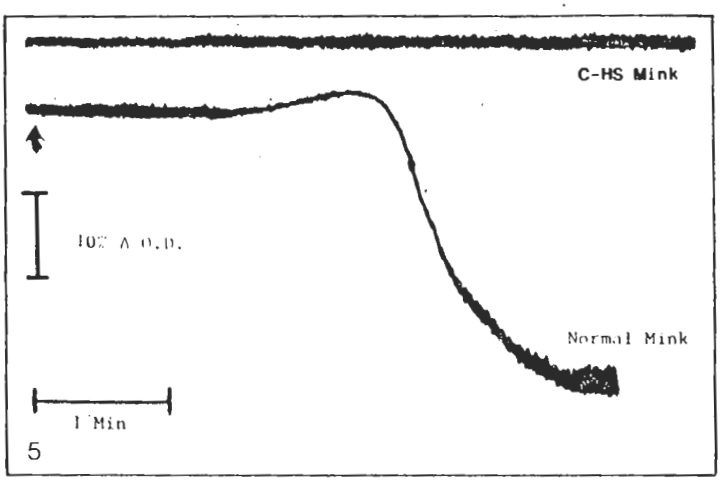

Fig 5—Aggregation curves in CHS-affected mink and normal mink, induced with collagen after in vivo treatment with indomethacin (addition at arrow).

for all groups. The ADP-induced aggregation rates for both affected and normal mink are indistinguishable. Collagen-induced aggregation rate for platelets from affected mink, 37.3 U/minute, is about half of the normal rate (73.0 U/minute), which is significantly different at $P < 0.01$. To determine whether this difference was due to a cyclo-oxygenase inhibitable function, the animals were treated with the cyclo-oxygenase inhibitors indomethacin and aspirin. The CHS-affected mink treated with these agents failed to aggregate in response to collagen stimulation. On the other hand, platelets from normal mink were only inhibited to half the normal rate (from 73.0 to 34.0 after the animals were given aspirin and to 46.0 U/minute after the animals were given indomethacin, $P < 0.01$). The ADP-induced aggregations remained at pretreatment rates, demonstrating consistency in sampling-aggregation methods. Six of 10 affected mink which were given cyclo-oxygenase inhibitors, 4 in the aspirin-treated group and 2 in the indomethacin-treated group, were dead within 30 minutes after the sampling procedure. At necropsy, they were found to have large fibrin thrombi within the pericardial sac. None of the normal mink (n = 10) administered the same dosage of drug died.

Discussion

Bleeding times in normal mink and in Aleutian mink affected with CHS have not been previously reported. How-

TABLE 2—Comparative Aggregation Response of Blood Platelets from CHS-Affected Mink

Mink No.	Status	Platelet concentration ($1 \times 10^3/\mu l$)	ADP aggregation rate (U/min)	Collagen aggregation rate (U/min)
Normal mink (n = 20)				
1	Before	ND	83.3	62.6
2	treatment	625	68.7	71.4
3		289	53.6	75.5
4		447	46.4	78.3
5		312	40.0	53.6
6		344	62.5	71.4
7		331	78.5	90.0
8		323	62.5	71.4
9		347	62.5	80.0
10		437	50.0	79.2
Mean (± SD)		384 ± 102	61.0 ± 13.7	73.0 ± 3.2
11	Treated	367	47.5	21.4
12	with	440	62.5	43.8
13	indomethacin	658	67.5	43.8
14		527	74.2	37.5
15		253	52.7	23.5
Mean (± SD)		449 ± 154	61.0 ± 11.0	34.0 ± 4.9
16	Treated	375	78.5	47.2
17	with	559	96.6	71.9
18	aspirin	308	53.5	40.0
19		372	50.3	45.8
20		640	43.7	25.0
Mean (± SD)		451 ± 141	65.0 ± 22.3	46.0 ± 7.6
Affected mink (n = 20)				
1	Before	375	37.5	30.0
2	treatment	477	90.0	60.7
3		636	65.0	47.5
4		608	62.5	50.0
5		218	42.7	12.5
6		485	80.0	57.1
7		671	54.2	13.7
8		325	40.6	20.8
9		336	62.5	33.3
10		287	69.4	47.2
Mean (± SD)		442 ± 143	60.4 ± 17.0	37.3 ± 5.6
11	Treated	492	70.0	≤ 0.1
12	with	289	35.0	≤ 0.1
13	indomethacin	418	102.5	2.8
14		532	84.0	≤ 0.1
15		348	116.5	10.6
Mean (± SD)		416 ± 100	82.0 ± 32.0	2.7 ± 4
16	Treated	626	80.0	≤ 0.1
17	with	303	18.0	≤ 0.1
18	aspirin	256	39.3	≤ 0.1
19		244	28.0	≤ 0.1
20		418	35.5	≤ 0.1
Mean (± SD)		369 ± 159	40.2 ± 24.0	0.1 ± 0

The values are means of samples, one sample per mink. ND = not determined.

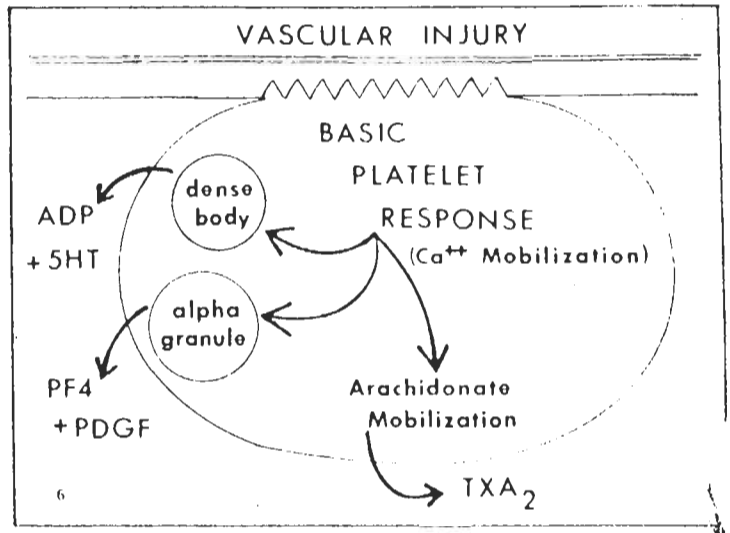

Fig 6—Normal platelet response at the site of vascular injury is a consequence of subendothelial collagen exposure, and is characterized by basic response with shape change, release of dense granule adenosine diphosphate (ADP) and serotonin (5HT), α-granule release of platelet factor 4 (PF4), and platelet derived growth factor (PDGF), and arachidonic acid mobilization from membrane with production of thromboxane A_2 (TXA_2). Results of this investigation indicate that the platelets from CHS-affected mink have no dense-body release, but that arachidonate metabolism is normal (α-granule function was not investigated).

ever, fatalities which can be attributed to inadequate hemostasis are a common occurrence in Aleutian mink herds after nail clipping, tail biting, and trauma. Platelet dysfunction has been related to long bleeding times in many other species and this study clearly demonstrates increased bleeding time in CH mink.[15,16]

Platelet dysfunction produces a defect in primary hemostasis, the formation of the initial hemostatic plug. This type of prolonged bleeding is commonly a result of one or any combination of the following platelet abnormalities: (1) a numerical decrease in platelets within the circulation, (2) a reduced platelet adherence or "adhesiveness," (3) an inhibition of arachidonic acid metabolism reducing thromboxane A_2 production, and (4) a deficit in the dense body (nucleotide/serotonin) release. Inhibition of arachidonate metabolism is believed to decrease dense body release secondarily. Examples of clinical disorders corresponding to these abnormalities of platelets are (i) thrombocytopenic purpura, (ii) von Willebrand's disease, (iii) aspirin-induced thrombocytopathy, and (iv) CHS of Aleutian mink, Hereford cattle, Persian cats, and persons.

The nucleotide data from the present study confirm that the granule (storage pool) ADP is deficient. Metabolic nucleotides, however, are known to be normal in CHS in other species and presumably are similarly unaffected in the mink. While we do not present specific data in this regard, for affected mink the platelet response to ADP is normal. This fact is indirect evidence that no platelet defect in nucleotide energy metabolism is present since, in normal platelets, inhibitory agents affecting metabolic nucleotides produce a dysfunction in ADP-induced aggregation.[18,21,22]

In terms of the mink primary hemostatic apparatus, we can now ask how important the secretion of stored nucleotide compared with prostaglandin production is for platelet function. Our examination of platelet function after cyclo-oxygenase inhibition (effectively blocking thromboxane A production) indicates that roughly half the aggregability in response to collagen ex vivo depends on nucleotide secretion. This conclusion is based on the fact that blocking the cyclo-oxygenase pathway in platelets from CHS-affected mink resulted in functional failure, whereas untreated platelets from the same mink had roughly half normal aggregability. The in vivo significance is underscored by the fact that 60% of the cyclo-oxygenase-treated mink with CHS died after cardiac puncture.

Platelets have a key role in the response to injury within blood vessels. The repeated release of a platelet-derived growth factor stored within the α-granule has been shown to result in arteriosclerosis in some animals.[23] Although factors which affect storage pool and α-granule release are not completely understood, the severe platelet disorder in CHS-affected animals will enable investigation of the phenomenon. The possibility of CHS conferring resistance to arteriosclerosis needs to be tested (Fig 6).

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