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Ultrastructural aspects of captive hooded seal (*Cystophora cristata*) platelets

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Abstract

To determine the role of platelets in normal haemostasis and pathological atherosclerosis, specific aspects of their ultrastructure have been investigated. Pinnipeds have been used as a model to study the dietary effects of marine oils, rich in n-3 fatty acids, on the reduced incidence of human cardiovascular disease.

Ultrastructural features of hooded seal (*Cystophora cristata*) platelets resemble those characteristic of circulating mammalian thrombocytes. These include: submembrane filaments, a circumferential band of microtubules, a surface-connected canalicular system, a dense tubular system, alpha granules, mitochondria, dense bodies and glycogen. Hooded seal platelets, however, were found to remain discoid and maintain their circumferential band of microtubules when cooled to and fixed at 4°C.

Introduction

There has been a dramatic rise in the incidence of human cardiovascular disease in the latter half of the 20th century and a corresponding increased appreciation that blood platelets play an important role in haemostasis and atherosclerosis. These changes have triggered a demand for knowledge regarding platelet morphology, physiology and function (White, 1979). Extensive research has been conducted involving blood platelets of man and many mammalian species (Maupin, 1969). There are few data, however, regarding pinniped platelets.

Mammalian platelets are fairly conservative in morphology and ultrastructure (Robinson *et al.*, 1969; Lewis & Bowie, 1978; White, 1979) with characteristic structures such as a circumferential band of microtubules, microfilaments, dense bodies, granules, mitochondria, glycogen, a dense tubular system and a surface-connected canalicular system (White, 1979; Meyers *et al.*, 1982). Electron microscopy has provided valuable information defining many structural elements involved in the haemostatic function of blood platelets (White, 1987).

Epidemiological studies have indicated an association between the low incidence of acute myocardial infarction amongst the Greenland Inuits (Bang *et al.*, 1976; Dyerberg *et al.*, 1975; Dyerberg & Bang, 1979) and Japanese coastal fisherman (Hirai & Tamura, 1987) with their high dietary intake of n-3 fatty acids. Also, a large amount of evidence has accumulated linking the ingestion of marine oils rich in eicosapentaenoic acid (20:5(n-3), EPA) and docosahexaenoic acid (22:6(n-3), DHA) with prolonged bleeding times, diminished platelet response to agonists and decreased risk of atherosclerosis and ischaemic heart disease (Sanders *et al.*, 1980; Siess *et al.*, 1980; Ahmed & Holub, 1984).

Recent research has introduced pinnipeds as animal models for studying the effects of dietary fats on platelet aggregation (Puppione *et al.*, 1987; Ahmed *et al.*, 1989; Miller, 1989). Since most pinniped species subsist solely on marine organisms (King, 1983), rich in n-3 fats, they may provide a valuable model to study the long term effects of a diet rich in omega 3 polyunsaturated fatty acids on platelet composition, activity and the physiological mechanisms that give rise to favourable platelet function in terms of reduced cardiovascular disease (Puppione *et al.*, 1987). Pinnipeds appear to be free of atherosclerosis (Ackman *et al.*, 1979), exhibiting prolonged bleeding times in air and platelets which are unstimulated by agonists at levels which cause aggregation of human platelets (Puppione *et al.*, 1987). The ultrastructures of resting seal platelets are presented here for the first time.

Materials and Methods

One adult female and two male hooded seals (*Cystophora cristata*) housed at the University of Guelph, were maintained in freshwater flow through tanks at a temperature of approximately 10°C. The animals were fed twice daily on a diet of whole

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herring (*Clupea harengus*), supplemented with sodium chloride and vitamins.

For each animal, 30 ml of blood were collected from the hind flipper plexus (Ronald *et al.*, 1969; Geraci, 1971) with siliconized Vacutainer tubes containing 1/6 volume of 1.5% Na_2EDTA, 0.85% NaCl as anticoagulant (Holub & Celi, 1984). The blood was spun in a centrifuge at 300 g for 15 min at 4°C. The platelet rich plasma was drawn off with siliconized Pasteur pipettes and suspended in a 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.4) at a volume ratio of 1:20. After fixation for one hour at room temperature, the cells were respun at 300 g for 10 min and the fixative was removed. The pellet was transferred to a Pyrex tube and post-fixed in 1.0% osmium tetroxide in phosphate buffer and dehydrated in an ascending ethanol series. The pellet was infiltrated with Epon via propylene oxide, flat embedded in aluminium weigh boats and polymerized at 60°C for 24 hours. Ultrathin sections were cut on a L.K.B. Ultratome and mounted on uncoated 100 mesh copper grids. Sections were double contrasted in 2% ethanolic uranyl acetate and lead citrate and viewed on a JEOL JEM100CX transmission electron microscope.

Results

Through electron microscopic examination, it was evident that after fixation hooded seal platelets retained structural characteristics that are typical of mammalian platelets. The thrombocytes appeared as enucleated discoid cells showing heterogeneity in both size and shape (Figs 1, 2, 3). Submembrane filaments, close to the innerside of the plasma membrane were evident in the equatorial section (Fig. 3). Microfilaments were observed in the pseudopodia of platelets that had changed shape (Fig. 2). The serpentine-like canaliculi appeared as continuous invaginations of the platelet plasma membrane (Figs 2, 3). Although they occurred randomly throughout the cell, most appeared in close proximity to granules and other platelet organelles. Microtubules were evident in both the annular form (Fig. 3) and cross-section just beneath the cell membrane (Fig. 2). The alpha granules, varying in density, size and shape were apparently the most abundant of the platelet organelles (Figs 1, 2, 3). The so-called 'Bull's eye' granules (Keyhani, 1970) of dense bodies were observed in their typical form, consisting of a very dark sphere in a clear circular membranous structure (Fig. 3). The platelet mitochondria were evident and could be differentiated from alpha granules by the characteristic invaginations or cristae (Fig. 2).

Elements of the dense tubular system were evident in association with the circumferential band of microtubules and channels of the surface-connected canalicular system (Figs 2, 3). Glycogen granules were observed as discrete particles and as small masses in all platelets observed (Fig. 2).

Discussion

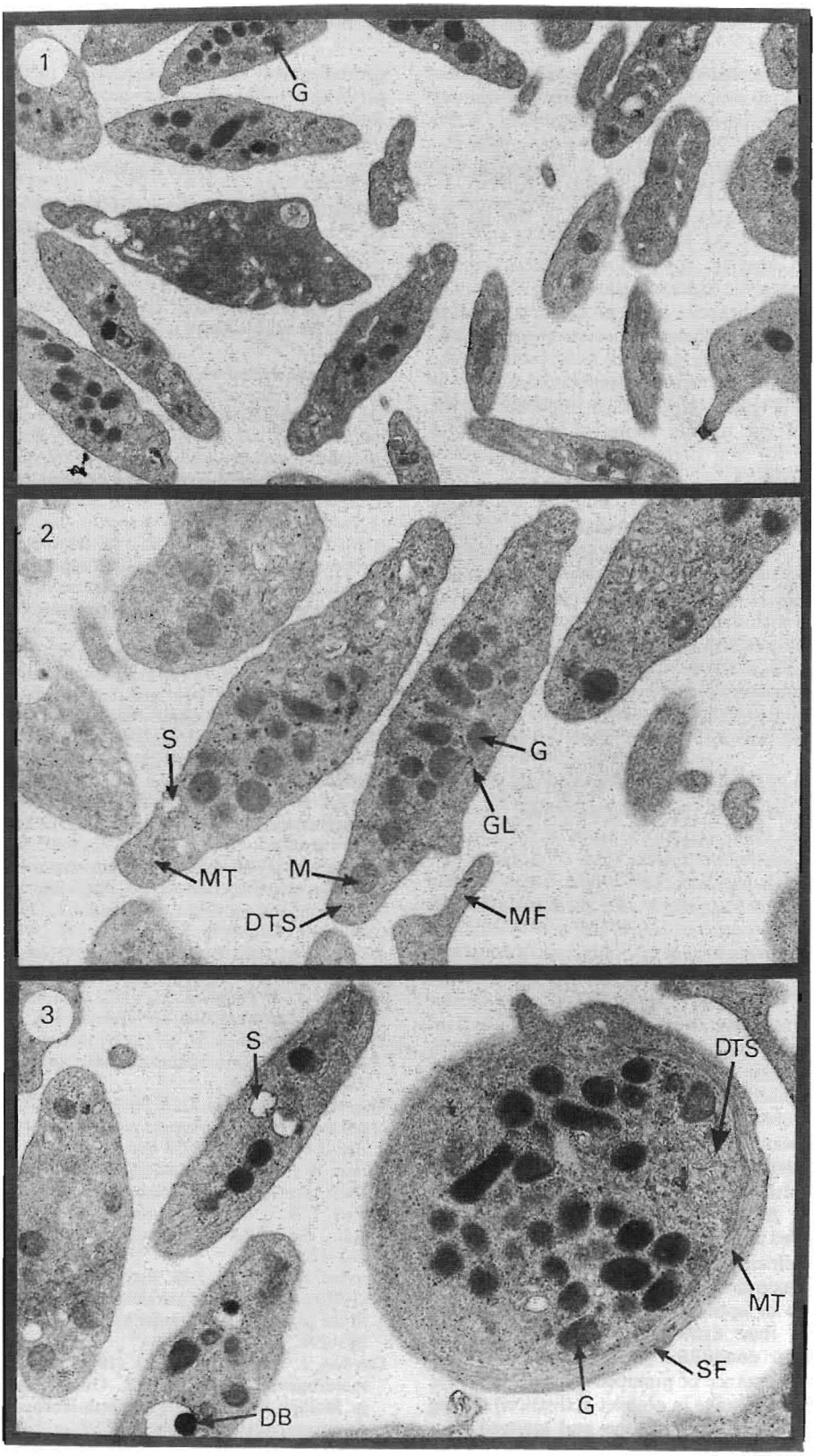
Hooded seal platelet ultrastructure can be described according to White's (1971b) scheme of anatomical zonation which divides the cell into four regions.

The peripheral zone, involved in adhesion and aggregation consists of the plasma membrane and the exterior coat or glycocalyx.

The sol-gel zone, which is vital for cytoskeletal support and cell contraction, consists of the cell cytoplasm or matrix and its fibre systems including microtubules and microfilaments. Submembrane filaments were evident in hooded seal platelets and were located in close proximity to the inner side of the plasma membrane (Fig. 3). Their main function is to maintain the discoid shape of platelets and aid in the formation of pseudopods upon activation (Zucker-Franklin, 1970; White & Gerrard, 1980; White 1987). Annular bands of microtubules were evident in both the equatorial (Fig. 3) and cross (Fig. 2) sections of the hooded seal platelets. These structures along with the cytoskeletal submembrane actin filaments are important in maintaining the discoid shape of resting cells (Behnke, 1965; Gordon & Milner, 1976; White, 1987). They also participate in the process of internal reorganization of the cell's organelles when exposed to agonists (White, 1971a).

The organelle zone, involved in storage and secretion, includes the cell's organelles. Clearly the most striking feature of mammalian platelets is the specific granules or alpha granules (Figs 1, 2, 3). They primarily serve to store components involved in platelet aggregation, such as fibrinogen, von Willebrand factor, and platelet factor 4 (antiheparin factor) (Day & Holmsen, 1971; Firkin, 1984). Numerous dense bodies are also apparent in the

Figures 1–3. Electron micrographs are of female hooded seal (83–5) platelets spun and fixed at 4°C. Platelets appear unstimulated, showing normal discoid shape and ultrastructural features. Fig. 1. Cross-section of seal platelets (8300 ×). Most platelets are discoid in shape, displaying numerous alpha granules (G). Fig. 2. Cross-sections of seal platelets (13 000 ×). The following ultrastructural features are demonstrated: the surface-connected canalicular system (S), microtubules (MT), alpha granule (G), microfilaments (MF), mitochondria (M), dense tubular system (DTS), and glycogen (GL). Fig. 3. Seal platelets are discoid with one cut in the equatorial plane (13 000 ×). Ultrastructural features include: submembrane filaments (SF), surface-connected canalicular system (S), annular ring of microtubules (MT), alpha granule (G), dense body (DB) and dense tubular system (DTS).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hooded seal platelets (Fig. 3). In mammalian platelets they provide a second storage system, containing serotonin, adenine nucleotides and calcium (White & Gerrard, 1980). The opacity of the dense granules is due to the presence of high concentrations of calcium, ATP and ADP (Sixma, 1986).

Mitochondria were also present in the phocid platelets (Fig. 2). As has been described in other mammalian species, the mitochondria exhibited their characteristic structure (Firkin, 1984). They function, as in other cells, in the production of energy through oxidative phosphorylation (Caen *et al.*, 1977). Many glycogen granules were visible in the seal platelets and occurred as discrete granules or as small lakes (Fig. 2). Glycolysis is important in the retention of the discoid shape of platelets, thus accounting for the presence of this component (Behnke, 1970).

Finally the membrane systems are comprised of the surface-connected canalicular system and the dense tubular system. The surface-connected canalicular system was evident in hooded seal platelets (Figs 2, 3). These cellular invaginations are continuous with the plasma membrane (Fig. 2) and increase the surface area exposed to the plasma (Sixma, 1986). They provide a mechanism for the uptake of substances from the plasma and the extrusion of endogenous components secreted during the cell's release reaction (White, 1968; Holme *et al.*, 1973).

Elements of the dense tubular system were evident in association with the circumferential band of microtubules (Fig. 2) and channels of the surface-connected canalicular system (Fig. 3) (Caen *et al.*, 1977; White & Gerrard, 1977). The dense tubular system has an important role in prostaglandin production (Gerrard *et al.*, 1976) and in calcium sequestration which can be mobilized during the release reaction and aggregation (Firkin, 1984; White, 1987). Although substantial data are necessary to determine the biochemical and physiological composition of the hooded seal platelets, it is evident that they contain ultrastructural characteristics that are quite similar to other mammalian thrombocytes.

One platelet characteristic, perhaps unique to pinnipeds, was noted in this study. Although platelets from most mammals apparently lose their microtubule structures and discoid shape when cooled to 4°C (White & Krivit, 1967; White, 1968; Sixma, 1986), hooded seal platelets treated this way not only maintain their circumferential band of microtubules, but also remain discoid. Seals maintain a body core temperature of approximately 37°C, but the temperature of their extremities may approach 4°C during winter conditions (Harrison & Kooyman, 1968). The resistance of pinniped platelets to shape change (an early stage in platelet activation) during periods of restricted circulation and lowered temperatures may therefore be critical to the animals' survival.

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