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Unusually high concentrations of sialic acids on the surface of vascular endothelia

G.V.R. Born and W. Palinski
Department of Pharmacology, King's College, Strand, London WC2R 2LS

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Summary. Sialic acids present on luminal surfaces of vascular endothelium were determined by perfusing neuraminidase free of proteolytic activity through carotid arteries, iliac arteries and jugular veins of anaesthetized rabbits and guinea-pigs and through human umbilical veins. Total sialic acids released in 1 h from arteries and veins, determined fluorimetrically, were $24–51 \times 10^6$ molecules/μm^2 endothelial surface; this was more, by up to two orders of magnitude, than sialic acids releasable by neuraminidase from other types of cells, i.e. from 0.15×10^6 for human erythrocytes to 15×10^6 for human platelets. Sialic acids therefore provide extraordinarily high negative charge densities on vascular surfaces exposed to the flowing blood. As all circulating cells are also negatively charged, strong electrostatic repulsion must exist between them and normal vessel walls. These observations can therefore account for the general property of non-adherence of circulating cells in normal blood vessels of which the so-called 'non-thrombogenicity', meaning the non-adherence of platelets to normal vascular endothelium, is one example. It is suggested that a major biological function of these extraordinarily high negative charge concentrations is the mutual repulsion between endothelial surfaces and blood cells which promotes their unimpeded circulation.

Keywords: sialic acids, vascular endothelia

While demonstrating that the movement of plasma low-density lipoproteins into arterial walls is greatly accelerated by removal of their luminal sialic acids, we obtained evidence that the concentration of sialic acids is much greater on the surface of rabbit carotid endothelium than on that of other types of cell (Görög *et al.* 1982; Görög & Born 1982). This discovery has now been confirmed for other vascular endothelia. Our new evidence supports the proposition (Sawyer & Pate 1953; Sawyer & Srinivasan 1972; Thubrikar *et al.* 1980) that the non-adherence of circulating cells in normal vessels is due to electrostatic repulsion between the negatively charged surfaces of the vascular endothelium and the blood cells.

Methods

Determination of sialic acids. Removable sialic acid was determined by perfusing neuraminidase free of proteolytic activity through carotid arteries, iliac arteries and jugular veins of anaesthetized rabbits and guinea-pigs and through human umbilical veins. Total sialic acid in the perfusates of arteries and veins was determined fluorimetrically (Hess & Rolde 1964). Perfusate (200 μl) was added to an equal volume of 5 μM 3,5-di-

aminobenzoic acid (DABA) in 0.125 N HCl. The mixture was sealed in a 2-ml ampoule and heated at 110°C for 16 h. A 50-μl sample was then transferred into 1.5 ml of 0.05 N HCl and its fluorescence was determined with a Perkin-Elmer fluorescence spectrometer MPF4, using an excitation wavelength of 405 nm and recording emission between 460 and 530 nm.

Perfusion of rabbit carotid arteries and jugular veins. Male rabbits weighing 3.5–4.2 kg were anaesthetized with pentobarbital (35 mg/kg i.v.) and additional ether. Both carotid arteries and jugular veins were exposed for about 3 cm where branches were tied off; blood flow was then interrupted by clamping both ends. The proximal and distal ends of the segments were cannulated with fine needles (30G × 20). Both inflow and outflow cannulas passed through a perfusion pump (Watson–Marlow 501), so that the pressure in the perfused segments was constant. After perfusing first with phosphate-buffered saline at pH 7.4 to remove the blood, perfusion was continued for 60 min with buffered saline containing neuraminidase (Vibrio cholerae, protease-free, from Behring Werke) at a concentration of 0.05 U/ml and at a flow rate of 5 ml/h. Fractions of the perfusate were collected every 10 or 20 min; their volumes were established by weighing and their sialic acid contents determined fluorimetrically as described above. Control perfusions were without neuraminidase for similar periods. Guinea-pig carotid arteries were perfused similarly.

Perfusion of human umbilical cord veins. Human umbilical cords obtained from normal or caesarean deliveries were thoroughly rinsed inside and out with phosphate-buffered saline to remove blood. Each cord was cut into segments about 5 cm long which were kept in saline at 37°C. The umbilical vein was cannulated with a special teflon nozzle and perfused at 10–12 ml/h, first with saline only for 20 min to remove all remaining blood and then with neuraminidase. The time from obtaining the cord and beginning the perfusion of the last segment did not exceed 20 min. After perfusion, the veins were filled with glutaraldehyde solution at 5–10 mmHg for 15 min; they were then isolated from the cord and fixation was continued as already described. The tortuosity of the veins required some of them to be further subdivided before their surface areas could be determined.

Determination of lumenal surface area. The vascular segments were perfused for 15 min with 2.5% glutaraldehyde at a pressure of 100 mmHg for arteries and of 10 mmHg for veins; this prefixation proved to be essential for determination of the luminal surface areas. The fixed segments were cut off, opened longitudinally and the ends beyond the perfusion needles were removed. The open vessels were pinned out flat without tension and fixation was continued for another 12 h. Luminal surface areas were measured with a photographic magnifier. The image of each segment, clamped between two microscope slides, was projected onto a sheet of paper on which its outline was traced and cut out. The weight of the traced area was compared to that of a reference area of 100 mm^2.

Results are expressed as number of molecules of sialic acids per square micrometre of endothelial surface.

Control of endothelium after perfusion. The condition of the endothelium in vessels that had been perfused was assessed by silver staining of intercellular junctions for light microscopical determination of endothelial intactness (Lautsch *et al.* 1953), by scanning electron microscopy (Fig. 1) and by microscopic counting of detached endothelial cells in centrifuged perfusates.

Results

To control our technique, particularly the activity of the enzyme preparation, its effectiveness in releasing sialic acids from the

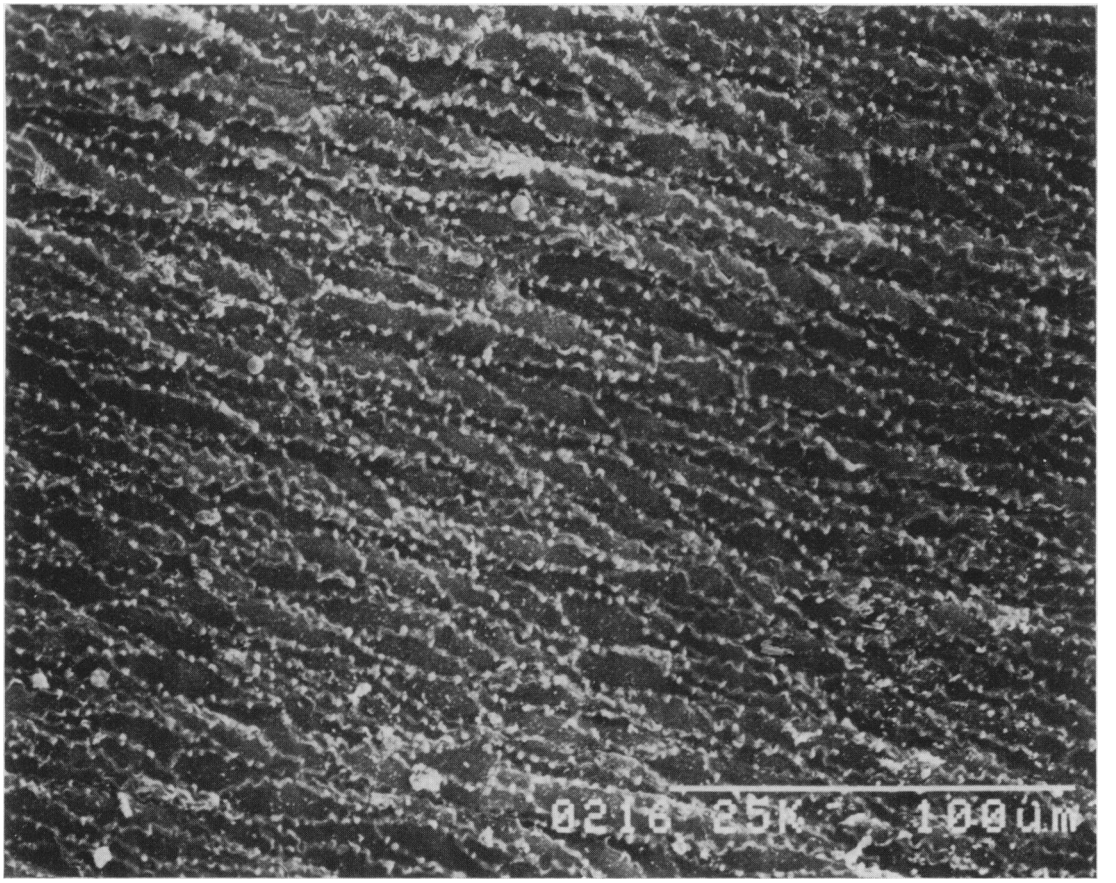

Fig. 1. Rabbit carotid artery endothelium after perfusion with neuraminidase for 1 h. Stained with $AgNO_3$ for intercellular junctions. Scanning electron microscopy. The scale bar represents 100 μm.

Table 1. Release of endothelial sialic acids by perfusing arteries and veins with neuraminidase

Blood vessel	Number of vessels	Sialic acids (pmol/mm^2 endothelial surface released after		
		20 min	40 min	60 min
Rabbit				
Carotid artery	10	31±3	49±3	63±5
Iliac artery	2	52	67	85
Jugular vein	10	28±3	46±3	60±3
Guinea-pig carotid artery	7	13±1	29±4	41±5
Human umbilical cord vein	10*	18±2	37±3	54±3

* Two segments were determined for each cord.
Results are mean ± SEM.

Table 2. Concentration of sialic acids removable by neuraminidase from the surface of different cells

Species	Cell type	Sialic acid concentration (10^6 molecules/μm^2)	Reference
Rabbit	Carotid arterial endothelium	37.8	This paper
	Iliac arterial endothelium	50.9	" "
	Jugular venous endothelium	35.9	" "
Guinea-pig	Carotid arterial endothelium	24.4	" "
Human	Umbilical venous endothelium	32.8	" "
	Erythrocytes	0.18	Our data
		0.15	Eylar *et al.* (1962)
		0.26	Massamiri *et al.* (1979)
	Platelets	15.0	Peerschke & Zucker (1978)
	Lymphoid cells	0.4–2.4	Rosenberg & Einstein (1972)
Mouse	Ascites tumor cell	3.0	Codington *et al.* (1979)

surface of intact human erythrocytes was determined for comparison with published values. Sialic acids releasable by our neuraminidase from human erythrocytes were 48 ± 16 nmol/10^9 cells (mean $\pm$ SEM, $n = 7$), similar to 60 nmol/10^9 cells determined previously (Massamiri *et al.* 1979). This gave confidence that our enzyme was active and our quantification technique in order.

Perfusion with neuraminidase under the conditions described released large quantities of sialic acids from all the blood vessels so far investigated (Table 1). In three vessels from rabbits, viz carotid and iliac arteries and jugular vein, considerably more sialic acids were released in the first 20 min than in the two subsequent 20-min periods. In guinea-pig carotid and human umbilical vein the initial release was similar but did not decrease noticeably with time. Because of this, in some experiments exposure to neuraminidase was continued; after about 2 h there was a steep increase in sialic acid release.

Total sialic acids released from vessels in the three species in 1 h were similar i.e. $24–51 \times 10^6$ molecules/μm^2 endothelial surface (Table 2). These values were all higher than corresponding values for other types of cell, varying from about two-fold compared with human platelets to about 200-fold compared with human red blood cells.

Discussion

The results show that the luminal surface of the endothelium of large veins and arteries has extraordinarily high concentrations of sialic acids. The endothelial sialic acids released by neuraminidase are presumably terminal constituents of glycoproteins, so that our observations are compatible with high concentrations of endothelial surface glycoproteins, including fibronectin (Wagner & Hynes 1979).

In all three rabbit vessels, release of sialic acids was greatest in the first 20 min and less in subsequent similar periods, as might have been expected. In guinea-pig carotids and human umbilical veins, release was more or less similar in all three periods. The reason for this is not known. When perfusion with neuraminidase was continued, after about 2 h there was a steep increase in sialic acid release, confirming previous observations (Görög & Born 1982). This increase is apparently associated with the ability of the enzyme to act beyond the endothelial surface (Görög & Pearson 1985).

Sialic acids thus provide an exceptionally high density of negative charges on vascular surfaces exposed to the flowing blood. That the intimal surface of large blood vessels is negatively charged has been known for some time (Sawyer & Pate 1953; and also that

artificial reduction of this charge is thrombogenic (Sawyer & Srinivasan 1972). These observations gave rise to the proposition (Sawyer & Pate 1953; Sawyer & Srinivasan 1972) that the so-called 'non-thrombogenicity' of normal vascular endothelium is primarily attributable to electrostatic repulsion between the endothelial surface and that of platelets, the surface negativity of which was also established some time ago (Madoff *et al.* 1964; Boisseau *et al.* 1977).

Previous evidence for the existence and magnitude of the net negative charge on vascular endothelia depended mainly on determination of artificially induced streaming potentials, from which the zeta potentials and the corresponding surface charge density were calculated (Thubrikar *et al.* 1980). Our observations are the first direct experimental evidence for at least one and probably the predominant origin of the overall net negativity of endothelium.

It is interesting to compare the charge densities calculated from these two different experimental approaches. From measurements of the streaming potential in dog femoral arteries, the surface charge density came out as $0.3–1.8 \times 10^5$ electrons/μm^2. This calculation depends on several assumptions, the combined effect of which is to make the value an underestimate by at least one order of magnitude (Thubrikar *et al.* 1980). From our determinations of sialic acids removable by neuraminidase from arterial and venous endothelia, the surface charge density works out as $2.4–5.1 \times 10^7$ electrons/μm^2. For a variety of other types of cell, neuraminidase-labile sialic acids account for 70–80% of the total surface negativity (Seaman & Cook 1965). If the surface of endothelial cells is like that of all other cells so far investigated, the net negative charge that determines electrophoretic mobility is accounted for almost entirely by neuraminidase-labile sialic acids. These considerations suggest that the discrepancy between the two results is much smaller than the figures indicate; what difference remains may be accounted for by the binding of counterions.

As all types of cells circulating in the blood are also negatively charged, it can be inferred that strong electrostatic repulsion must exist between normal vessel walls and circulating cells. Our discovery therefore supports the proposition (Oka 1983) that strong electrostatic repulsion accounts for the general non-adherence of circulating cells in normal blood vessels of which the so-called 'non-thrombogenicity', meaning the non-adherence of platelets to normal vascular endothelium, is only one example. This non-adherence of platelets has been widely attributed to the ability of normal vessels to produce prostacyclin (Moncada & Vane 1979; Moncada *et al.* 1977). However, the adhesion of platelets to vessel walls is not increased when their prostacyclin production is selectively inhibited (Dejana *et al.* 1980) whereas adhesion is increased when the endothelial surface is selectively desialated (Görög *et al.* 1982). Furthermore, circulating blood contains no prostacyclin (Blair *et al.* 1982; Dollery *et al.* 1983) and, in any case, it could not explain the non-adherence of other blood cells, particularly of the erythrocytes.

The interaction represented by this electrostatic repulsion is strong but, of course, entirely non-specific. Our observation therefore serves to increase interest in the mechanisms responsible for the specific interactions between blood vessel walls and different types of circulating cell upon which their physiological functions depend, i.e. polymorphonuclear leucocytes (granulocytes) in inflamed venules; small lymphocytes in post-capillary venules of lymphoid tissues; and platelets in acutely damaged vessels of all kinds (Porter *et al.* 1980). In these interactions the different blood cells adhere to the vessel walls, implying the existence of processes capable of overcoming the strong electrostatic repulsion forces normally present.

If it turns out, from experiments under way, that endothelium of the microcirculation also has such extraordinarily high negative charge densities, they would constitute one of the properties required for explaining

the ability of erythrocytes to squeeze through capillaries, the other property being red cell deformability (Bessis *et al.* 1978).

We suggest that a major biological function of these extraordinarily high negative charge concentrations is the mutual repulsion between endothelial surfaces and blood cells which promotes their unimpeded circulation.

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