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Short Communications

Membrane Glycopeptides from Old and Young Human Erythrocytes

By Cesare Balduini, Carlo Luigi Balduini and Edoardo Ascari
Institute of Biological Chemistry, Faculty of Sciences, and Institute of Clinical Medicine, Faculty of Medicine and Surgery, University of Pavia, 27100 Pavia, Italy

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Glycopeptides were extracted by papain digestion from old and young human erythrocyte membranes and fractionated on DEAE-Sephadex A-25. Chemical characterization of the unfractionated samples and of the main peak eluted from the column indicates that glycoproteins of the erythrocyte membrane undergo significant decreases in sialic acid and galactosamine content with aging.

Glycoproteins in human erythrocyte membranes are mostly located at the external surface and are oriented with the *N*-terminal end to the exterior, whereas the *C*-terminal segment interacts with the membrane or is exposed to the interior of the cell (Marchesi *et al.*, 1972; Morawiecki, 1964; Winzler, 1969; Segrest *et al.*, 1973). As a consequence of this orientation glycoproteins can assume their fundamental role of receptor sites, thus regulating the interactions of the cell with the external environment (Kraemer, 1971; Winzler, 1972). On the basis of the biological significance of membrane glycoproteins, and considering that human erythrocytes at the end of their life-span (about 120 days) are sequestered, particularly by the spleen, and are consequently destroyed, it was decided to investigate whether during aging of erythrocytes some modification of membrane glycoproteins occurs. It is well known that during their life-span erythrocytes undergo significant metabolic modifications (Fornaini, 1967); an alteration of membrane structure could be correlated with these events and could result in the inability of the erythrocyte to escape sequestration by the spleen and, consequently, lysis of the cell.

Experimental

Fresh human blood of the O Rhesus-positive group was collected from the veins of donors by using 3.8% (w/v) sodium citrate as anticoagulant. Plasma and buffy coat (white cells) were removed after centrifugation at 4°C at 1000*g* for 15min and the erythrocytes were washed three or four times with 10vol. of iso-osmotic (310mosM) sodium phosphate buffer, pH7.4 (Dodge *et al.*, 1963). The packed erythrocytes were then suspended in 0.9% NaCl.

Separation of old from young erythrocytes. The method based on the different osmotic fragility of young and old erythrocytes was used (Simon & Topper, 1957). Washed erythrocytes were suspended in 4vol. of 0.55% NaCl and cooled to 4°C. This temperature was maintained for 30min and the suspension was centrifuged for 15min at 4°C at 1500*g* and the supernatant containing the 'ghosts' was collected. The unlysed erythrocytes were then suspended in 4vol. of 0.53% NaCl and treated as described above. This procedure was repeated, with gradually less concentrated NaCl solutions, until all the erythrocytes were haemolysed. The percentage of haemoglobin present at each step in the supernatant was determined by the method of Drabkin & Austin (1935).

The 'ghosts' of the first haemolysed erythrocytes, corresponding to 30–35% of the total erythrocyte population, were collected and termed 'old ghosts'; those of the last haemolysed erythrocytes (30–35% of the total) represented the 'young ghosts' fraction. The 'ghosts' were then sedimented by centrifugation at 20000*g* at 4°C for 40min and washed with 20mosM-sodium phosphate buffer, pH7.4 (Dodge *et al.*, 1963), until the supernatant was colourless, to remove as much haemoglobin as possible. A partial lipid extraction was carried out by suspending the sediment in 10vol. of ethanol–diethyl ether (3:1, v/v) kept stirring for 5h at 4°C (Rosenberg & Guidotti, 1968). This treatment was repeated twice and the 'ghosts' were then washed with 99% (v/v) ethanol and then ether.

Extraction of glycopeptides from old and young 'ghosts'. Membrane glycopeptides were solubilized by papain digestion in 0.1M-sodium acetate buffer, pH5.5, containing 5mM-EDTA and 5mM-cysteine at 65°C for 48h (Scott, 1960); about 1 unit of papain/mg of dry 'ghosts' was used (1 unit will hydrolyse 1μmol of α-*N*-benzoyl-L-arginine ethyl ester/min at pH6.2 at 25°C). After removal of the insoluble particles, the solution was dialysed against a large

Table 1. *Composition of the glycopeptide mixture isolated from young and old human 'ghosts'*

Analytical methods are reported in the text. Basic amino acids were not determined. Amino acid composition is expressed in residues per 1000 amino acid residues.

	Content* (g/100g)			Amino acid content (residues/1000 residues)	
	Young 'ghosts'	Old 'ghosts'		Young 'ghosts'	Old 'ghosts'
Protein (Lowry)	24.6±2.2	25.5±2.0	Asp	157	171
Hexose	23.2±0.1	23.2±2.2	Thr	135	118
Fucose	4.1±0.1	3.6±0.1	Ser	179	166
N-Acetylglucosamine	13.2±1.9	14.0±2.4	Glu	209	224
N-Acetylgalactosamine	5.1±0.1	4.4±0.1	Pro	43	49
Sialic acid	13.7±1.2	9.9±2.1	Gly	59	67
SO_4^{2-}	traces	traces	Ala	65	68
			Val	63	59
			Met	18	9
			Ile	31	27
			Leu	37	36

* These values are means, ±S.D., of four to six determinations.

Table 2. *Composition of the main glycopeptide fraction eluted from DEAE-Sephadex A-25*

Analytical methods are reported in the text. Only the more significant amino acids are listed in the Table. Values are given as means of three determinations on the same sample. Fractionation was repeated on small samples of young and old 'ghosts', and the same elution pattern (E_{280}) and very similar ratios between sialic acid, glucosamine and galactosamine were obtained.

	Content (residues/1000 residues)	
	Young 'ghosts'	Old 'ghosts'
Total amino acids*	258.5	215.3
Hexose	319.8	352.1
Fucose	63.6	70.4
N-Acetylglucosamine	188.1	232.4
N-Acetylgalactosamine	62.5	42.7
Sialic acid	107.5	87.1
Aspartic acid	34.3	31.2
Threonine	56.3	44.4
Serine	71.5	55.8
Glutamic acid	23.1	22.0

* Only acid and neutral amino acids were determined.

excess of water and glycopeptides were precipitated in the presence of 0.6M-sodium acetate and 0.5M-acetic acid at 4°C by the addition of 3 vol. of 95% (v/v) ethanol; 1g of dry 'ghosts', obtained from 450ml of blood, yielded approx. 40mg of glycopeptides.

Fractionation of the glycopeptides on DEAE-Sephadex A-25. A portion (10mg) of papain-digested glycopeptides was fractionated on a column (1cm×30cm) of DEAE-Sephadex A-25; after washing with 100ml of double-distilled water, elution was carried out with 0.25M-, 0.50M-, 0.75M- and 1M-NaCl (about 100ml of each solution). The E_{280} was monitored with the ISCO Absorbance Monitor UA4: 2ml fractions were collected. Those corresponding to each peak registered were pooled, dialysed against water, concentrated to a known volume and analysed.

Analytical methods. Hexosamines and amino acids were determined with the Hitachi Perkin–Elmer Liquid Chromatograph by the method of Moore & Stein (1951). Sialic acid was measured by the method of Svennerholm (1958) and fucose by the method of Dische & Shettles (1948). Hexoses were determined by the method of Trevelyan & Harrison (1952). Total protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and sulphate groups were determined by the method of Terho & Hartiala (1971).

Gas–liquid chromatography. Trimethylsilyl derivatives of neutral sugars were analysed as reported by Cetta *et al.* (1972).

Results

Chemical characterization of the glycopeptides isolated from old and young 'ghosts'. The carbohydrate moiety of the glycopeptides of the erythrocyte membrane is composed of sialic acid, glucosamine, galactosamine, fucose and hexoses. G.l.c. analyses showed that these latter are represented mainly by galactose and by smaller quantities of glucose and mannose.

The modifications appearing with aging are mainly concerned with a significant decrease in sialic acid

content and with an increase in glucosamine/galactosamine ratio. In the protein moiety the main amino acids are aspartic acid, threonine, serine and glutamic acid. A decrease in the hydroxy amino acids and an increase in the dicarboxylic amino acids was also observed in the glycopeptides extracted from the old 'ghosts' (Table 1).

Fractionation of the glycopeptides from old and young 'ghosts' on DEAE-Sephadex A-25. Elution of the DEAE-Sephadex A-25 column with double-distilled water and subsequently with NaCl solutions of increasing concentration resulted in the isolation of five peaks, in the samples from both old and young 'ghosts'. The first peak was eluted with water, the second and the third were eluted with 0.25M-NaCl, the fourth was eluted with 0.5M-NaCl and the fifth with 0.75M-NaCl. In the second peak about 50% of the starting material was present; smaller quantities were recovered in the other fractions.

Table 2 shows the composition of the principal peak obtained from young and old 'ghosts' samples. The main modifications correlated with age occurring in this fraction are a decrease in sialic acid and galactosamine and an increase in glucosamine content. In the protein core serine and threonine decrease in the old 'ghosts' sample.

The other peaks eluted from DEAE-Sephadex showed a quite different composition, but no significant differences were revealed between the samples from old and young 'ghosts'.

Discussion

The glycoproteins of the human erythrocyte membrane undergo significant chemical modifications with aging, which consist particularly of a decrease of sialic acid and galactosamine content.

It must be considered that probably the differences between the membrane glycoproteins of a very young and a very old erythrocyte are much more evident than those shown by our results; in our experiments, in fact, the young and the old 'ghosts' fractions each corresponded to about 35% of the total erythrocyte population and it was not possible in our work to isolate the cells just entered into the circulating blood from those that were going to be destroyed.

In the unfractionated sample from old 'ghosts' a decrease in sialic acid and galactosamine and an increase in glucosamine were observed; in the protein moiety a decrease in serine and threonine and an increase in aspartic acid and glutamic acid occur.

After separation on DEAE-Sephadex the main glycopeptide fraction eluted from the column shows, in the sample obtained from old 'ghosts', modifications similar to those mentioned above. It is remarkable in this case that the decreases in sialic acid and in galactosamine are equimolar. This evidence suggests that the modifications occurring during erythrocyte aging mainly consist of the removal from the glycopeptide of a disaccharide chain containing sialic acid and galactosamine. Sialic acid partially disappears also from the other membrane glycopeptides, and this concurs with the observation that in the unfractionated sample the decrease in sialic acid is more than that of galactosamine.

The role of sialic acid in the rate of turnover of plasma glycoproteins (Morell *et al.*, 1971) and in lymphocyte circulation (Woodruff & Gesner, 1969) is well established. It remains to be seen if a similar role can be ascribed in the circulation of erythrocytes.

On the basis of our results it may be hypothesized that the life-span of erythrocytes is determined by the disaccharide unit, sialic acid–galactosamine, present in membrane glycoproteins; its decrease below a certain threshold could inhibit the capacity of the erythrocyte to escape sequestration by the spleen and consequently could induce lysis of the cell. The modifications of membrane glycoproteins are probably correlated with the metabolic changes characteristic of erythrocyte aging, but so far the mechanism by which these modifications occur is completely unknown.

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