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Reduced diversity of the human erythrocyte membrane sialic acids in polycythemia vera. Absence of *N*-glycolylneuraminic acid and characterisation of *N*-acetylneuraminic acid 1,7 lactone

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Abstract

Sialic acids from the erythrocyte (RBC) membrane of a patient suffering from polycythemia vera, a malignant orphan disorder of hematopoietic cells, was studied using GC/MS. We found that the sialic acid diversity of these membranes was drastically reduced since only four entities were identified: Neu5Ac (91.5%) and its 1,7 lactone Neu5Ac1,7L (7.5%) which is absent in normal RBC, Neu4,5Ac$_2$ (0.50%) and Neu4,5Ac$_2$ 9Lt (0.50%); in normal RBC, Neu5,7Ac$_2$, Neu5,9Ac$_2$, Neu5Ac9Lt, Neu5Ac8S and Neu, as well as traces of Kdn, were also present. Neu5Gc and its *O*-alkylated or *O*-acetylated derivatives, which are considered by various authors as cancer markers, were not detected.
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Keywords: Sialic acid; Red blood cell; Polycythemia vera; *N*-Glycolylneuraminic acid; *N*-Acetylneuraminic acid 1,7 lactone

1. Introduction

Polycythemia vera (PV) is a malignant disorder of hematopoietic stem cells which is characterised by clonal myeloproliferation with increased production of morphologically normal mature red blood cells, white cells and platelets [1−5]. First described in 1892 by Vaquez [6] polycythemia vera is a disease with an incidence of at least 2 per 100,000 and is a trilineage hematopoietic cell hyperplasia [7,8]. However, erythrocytosis is the most prominent clinical manifestation that may be due to increased proliferation or decreased apoptosis of erythroid progenitors, or to delayed erythroid differentiation with an increased number of progenitor cell divisions [9]. Prolonged red cell survival, another theoretical cause of polycythemia, has not yet been demonstrated [10]. Recent investigations have focused on a number of molecules involved in signal transduction pathways mediated by erythropoietin (Epo) and other growth factors, but human erythroid malignancies (PV and erythroleukemia) are associated with erythropoietin-independent growth and differentiation [11,12]. Despite recent advances in the characterisation of the malignant PV clone, the molecular mechanism and the abnormalities associated with the development of this disorder remain unknown [4].

In a previous article [13], we have demonstrated that the sialic acids of human erythrocyte membranes showed a large diversity independent of blood groups. Indeed *O*-acylated-*N*-acetylneuraminic acids (acetylated, lactylated), *O*-methylated and *O*-sulphated were present at a significant level but *N*-glycolylneuraminic acid and its *O*-alkylated or *O*-acylated

Abbreviations: PV, polycythemia vera; Epo, erythropoietin; RBC, red blood cell; HFBAA, heptafluorobutyric anhydride; GC, gas chromatography; MS, mass spectrometry; Kdn, 3-deoxy-D-glycero-D-galacto-nonulosonic acid.

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derivatives were not detected. This last result is of interest since it confirms the view that Neu5Gc is absent from healthy human tissues [14]. In fact, humans are genetically unable to synthesize Neu5Gc due to an exon deletion/frameshift mutation of the human CMAH gene [15–17]. In addition, Varki et al. [18] have recently demonstrated that Neu5Gc and its derivatives characterised in human cells and tissues were of dietary origin and that their presence could be explained by the direct incorporation of Neu5Gc-containing sialic acids from an exogenous source. However, despite the absence of any known alternative pathway for the synthesis of Neu5Gc in humans, various groups using antibodies, lectins or GC/MS claim the expression of this compound in human tumours [19–26] and it was proposed that Neu5Gc could be considered as a cancer marker (for review see [27]). In addition, recent studies using lectins and antibodies have revealed altered *O*-acetylation patterns in malignancies: decreased 9-*O*-acetylation in colorectal cancer [28,29] and colonic adenoma, neoexpression of 4-*O*-acetyl-sialic acid in mucins of these tissues [30–32] (for review see [27]). In addition, some alterations in erythrocyte cell surface sialoglycoproteins or more precisely appearance of some *O*-acetylated sialoglycoproteins were suggested as a distinct marker for differentiation between several leukaemia erythrocytes [33]. These controversial results prompted us to analyse the sialic acids present in the membranes of polycytemia vera RBCs regarded as cancerous cells by applying a method recently developed in our laboratory based on the use of gas chromatography–mass spectrometry which provides reliable data for trace amounts of sialic acids [34] in contrast to techniques based on the use of antibodies and lectins.

2. Materials and methods

2.1. Chemicals

Standard Neu5Ac and Neu5Gc were purchased from Sigma (St. Louis, USA). Neu4,5Ac_2, Neu5,9Ac_2 and Neu5,8,9Ac_3 were a generous gift from Prof. Roland Schauer (Kiel University, Germany). Diazogen™ was from Aldrich (Milwaukee, WI, USA) and heptafluorobutyric anhydride (HFBAA; puriss. grade) from Fluka (Buchs, Switzerland). Heavy walled screw cap tubes (10 × 100 mm) and Teflon-lined caps (GL14) were from Schott (Mainz, Germany).

2.2. Isolation of human polycythemia vera RBC membranes

For the preparation of RBC membranes peripheral human blood was taken up on heparin from a patient with O^+ blood group suffering from PV. The diagnosis of erythrocytosis (hematocrit 66%) was established at the Ştefan Berceanu Center of Bucharest according to commonly accepted clinical and laboratory criteria [35]. The blood samples were washed three times by centrifugation (5 min, 1000 × *g* at 4 °C) with phosphate buffered saline (PBS) (25 mM sodium phosphate buffer pH 7.2 containing 150 mM sodium chloride) in order to eliminate platelets and leukocytes by pipetting the supernatant. RBC haemolysis was performed using a ten-fold dilution of PBS in cold water (39 ml per ml RBC). After vigorous agitation, the samples were centrifuged (20 min, 10,000 × *g* at 4 °C) and the pellet was washed three times in the lysis buffer and centrifuged under the same conditions. A last washing of the pellet was performed in cold water in order to remove salts. Preparations were considered suitable for analysis of sialic acids when the membrane pellet was white.

2.3. Liberation and analysis of sialic acids

All operations were performed in heavy walled Teflon-lined screw caps tubes. For the liberation of sialic acids, the samples were hydrolysed (105 min at 80 °C in 2 M acetic acid in water) and evaporated in a rotary evaporator. The samples were submitted to a two-step derivatisation procedure: methyl-esterification of the carboxyl group with diazomethane, followed by acylation of free alcohol and amino groups with heptafluorobutyric anhydride (Taking into consideration that these reagents are harmful to health, all operations were performed under a well-ventilated hood). The dry samples were supplemented with 200 µl of anhydrous methanol and 200 µl of a diazomethane solution in diethyl ether [34] and left for at least 4 h at room temperature after vigorous agitation. When the GC/MS analysis had to be performed, the samples were treated individually as follows: the samples were evaporated to dryness under a stream of nitrogen, then supplemented with 400 µl of acetonitrile and 50 µl of heptafluorobutyric anhydride and heated for 5 min at 150 °C in a sand bath. After cooling the samples were evaporated under a stream of nitrogen and taken up in 1 ml of acetonitrile, dried on calcinated calcium chloride [34] and 1 ml of hexane was added. The hexane upper phase was discarded and this partition was repeated twice. The acetonitrile lower phase containing the derivatives of sialic acids was concentrated under a stream of nitrogen and an aliquot of 1 µl was injected onto the Ross injector of the GC/MS apparatus. Because of the very high amount of glycoprotein-bound sialic acid in human RBC membranes, the optimal conditions of analysis should be performed starting with less than 1 mg of RBC membrane proteins. Otherwise, the quantity of reagents indicated above have to be increased proportionally to obtain a complete derivatisation and to avoid saturation of the detector response for Neu5Ac.

2.4. Gas-chromatography and mass-spectrometry

The GC separation was performed on a Carlo Erba GC 8000 gas chromatograph equipped with a 25 m × 0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 µm film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 260 °C and the samples were analysed using the following temperature program: 90 °C for 3 min and then 5 °C/min until 260 °C. The column was coupled to a Finnigan Automass II mass spectrometer (mass detection limit 1000) or, for mass larger than 1000, to a Riber 10-10H mass spectrometer (mass detection limit 2000). The

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