EXPERIMENTAL CELL RESEARCH 320 (2014) 258–268

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Research Article

Sialic acid attenuates puromycin aminonucleoside-induced desialylation and oxidative stress in human podocytes

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ARTICLE INFORMATION

Article Chronology:
Received 5 June 2013
Received in revised form
7 October 2013
Accepted 27 October 2013
Available online 4 November 2013

Keywords:
Podocytes
Sialic acid
Sialyltransferase
Oxidative stress

ABSTRACT

Sialoglycoproteins make a significant contribution to the negative charge of the glomerular anionic glycocalyx—crucial for efficient functioning of the glomerular permselective barrier. Defects in sialylation have serious consequences on podocyte function leading to the development of proteinuria. The aim of the current study was to investigate potential mechanisms underlying puromycin aminonucleosisde (PAN)-induced desialylation and to ascertain whether they could be corrected by administration of free sialic acid.

PAN treatment of podocytes resulted in a loss of sialic acid from podocyte proteins. This was accompanied by a reduction, in the expression of sialyltransferases and a decrease in the key enzyme of sialic acid biosynthesis *N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE). PAN treatment also attenuated expression of the antioxidant enzyme superoxide dismutase (mSOD) and concomitantly increased the generation of superoxide anions. Sialic acid supplementation rescued podocyte protein sialylation and partially restored expression of sialyltransferases. Sialic acid also restored mSOD mRNA expression and quenched the oxidative burst.

These data suggest that PAN-induced aberrant sialylation occurs as a result of modulation of enzymes involved sialic acid metabolism some of which are affected by oxidative stress. These data suggest that sialic acid therapy not only reinstates functionally important negative charge but also acts a source of antioxidant activity.

Introduction

Accumulating evidence suggests that glycosylation defects may mediate the development of proteinuria following podocyte injury. The surface of podocytes is richly decorated by a high concentration of negatively charged sialoglycoproteins, the most abundant of which, at least in the rat, is podocalyxin. This epithelial 'polyanion' is essential for the maintenance of normal epithelial organisation [1]. In its absence normal foot process structure and filtration slit organisation becomes distorted leading to the collapse of filtration barrier function

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http://dx.doi.org/10.1016/j.yexcr.2013.10.017

[2–6]. The importance of the role of sialic acid has been illustrated in experiments wherein removal of sialic acid residues by infusion of sialidase or neutralisation of surface sialic acid charge using polycations such as protamine sulphate has resulted in podocyte effacement and proteinuria [7,8]. Conversely, infusion of a sialylated glycoprotein into experimental animals with puromycin aminonucleoside (PAN) nephrosis, a model characterised by reduced sialylation, improved podocyte ultrastructure [9]. However, to date the mechanism underlying the desialylation process has not been delineated.

As well as its characteristic negative charge, sialic acid has a unique α-ketocarboxylic acid structure, which has been shown to chemically scavenge H_2O_2 [10]. Consistent with this antioxidant action is the observation that the highly sialylated glycoprotein mucin exhibits reactive oxygen species (ROS) scavenging activity [11].

PAN nephrosis is an experimental model of proteinuria initiated by oxidative stress [12] and characterised by desialylation of key glomerular proteins begging the question whether the two processes may be causally related.

Supplementation with the sialic acid precursor *N*-acetyl mannosamine (ManNAc) has previously been shown to correct desialylation and its consequent renal lesions [13,14]. We hypothesise that treatment with sialic acid itself, given its antioxidant properties, may be a more efficient treatment modality as it could both attenuate oxidative stress and resialyate vital glomerular sialoglycoproteins.

The present study aimed to delineate possible mechanisms of PAN-induced abberant sialylation and to ascertain whether sialic acid could be used as a treatment modality.

Materials and methods

Cell culture

The human podocyte line used in these experiments was generous gift from Professor M Saleem. It expresses a temperature sensitive SV40 promoter that allows proliferative, undifferentiated growth at 33 °C and differentiated growth at 37 °C. The cells were fed every 2 days with RPMI 1640 containing 10% FCS, 100 μg/ml penicillin and 100 μg/ml streptomycin, and ITS supplement (insulin, selenium, transferrin) (Invitrogen, UK). When confluent cells were transferred to 37 °C and allowed to differentiate for 10 days. The cell line has been previously extensively characterised and has been shown to express a number of podocyte specific differentiation markers [15].

Podocyte culture

Podocytes in 25 cm^2 flasks were treated with 5 or 10 μg/ml PAN (Sigma, UK)±20 μg/ml sialic acid (Sigma) or 40 u/ml superoxide dismutase (Sigma). The cells were incubated for 3 days after which the supernatants and monolayers were analysed.

TRIzol RNA

Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer's instructions.

RT-PCR

RNA was reverse transcribed with the AMV reverse transcription system (Promega, UK). PCR using specific primers was carried out using Platinum Taq polymerase (Invitrogen).

Primers were custom made by Invitrogen. 18s primers were from TaqMan® (PE Applied Biosciences, CA, USA) and were used as the house keeping gene to normalise for loading.

After initial denaturation for 4 min at 94 °C, PCR was carried out for the number of cycles indicated in Table 1: denaturation 30 s at 94 °C, annealing 1 min at the temperature indicated in Table 1, extension 2 min at 72 °C (with the exception of ST6GalNac1 which was 30 s at each stage). The final elongation step was carried out for 7 min at 72 °C. Touch-down PCR was used for mSOD; following initialisation at 94 °C for 4 min, cycling was initiated at 65 °C (2 cycles), then the annealing temperature was reduced in increments of 2 °C (2 cycles at each temperature), finishing with 18 cycles at 55 °C.

Protein assay

Protein concentrations were determined by BioRad DC protein assay (BioRad, UK) using BSA standards according to the manufacturer's instructions.

Western blotting

Cell lysates for Western blotting were prepared by direct addition of 750 μl reducing Laemmli buffer directly to the cell monolayers in 25 cm^2 flasks. The lysates were sonicated with two 5 s bursts and centrifuged at 13,000 rpm in a microfuge. Podocyte lysates were heated to 100 °C for 5 min then resolved on SDS-polyacrylamide gels of appropriate pore size. The gels were blotted onto nitrocellulose membranes for 1 h at 100 V. The membranes were blocked in 5% milk protein solution in Tris-buffered saline+0.05%Tween 20 (TTBS) after which they were washed and incubated with primary antibody overnight at 4 °C. After washing, the membranes were incubated with the appropriate horseradish peroxidise (HRP)-conjugated secondary antibody (Dako UK Ltd, Cambridge, UK). Protein bands on the membrane were detected using the Pierce SuperSignal

Table 1

Gene	Acession no.	Forward seq.	Reverse seq.	Cycles	Anneal (°C)
ST6Gal 1	003032	1160–1181	1531–1510	32	55
ST3Gal5	001042437	619–638	815–796	29	63
ST8Sia1	003034	710–729	866–847	35	64
ST6GalNac1	018414	739–767	1194–1167	35	55
ST6GalNac2	006456	417–436	530–511	35	64
Podocalyxin	005397	1006–1028	1298–1277	31	58
mSOD	000636	381–402	700–679	Touch down	