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Changes of protein glycosylation in the course of radiotherapy

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ABSTRACT

This is the first study of changes in protein glycosylation due to exposure of human subjects to ionizing radiation. Site specific glycosylation patterns of 7 major plasma proteins were analyzed; 171 glycoforms were identified; and the abundance of 99 of these was followed in the course of cancer radiotherapy in 10 individual patients. It was found that glycosylation of plasma proteins does change in response to partial body irradiation (~60 Gy), and the effects last during follow-up; the abundance of some glycoforms changed more than twofold. Both the degree of changes and their time-evolution showed large inter-individual variability.

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1. Introduction

Radiation has been always a very important factor influencing living organisms. Radiation comes from accidental, intentional or natural sources; we can differentiate environmental, industrial, occupational (in wars) radiation, radiological terror and medicinal radiotherapy.

In radiobiology there is an increasing interest in proteomics nowadays [1–3]. The response for radiation was often studied on the genome level. However DNA damage is only part of the picture; changes in cellular structure; in protein composition of cells and biological fluids are also needed to understand molecular mechanisms and micro-environmental consequences of radiation. Furthermore, radiation may induce changes in higher order organisms. Beside genetics, proteomics may offer new insights, describing new pathophysiological pathways or discovering new biomarker candidates [4–10] to assess the influence of radiation on living, multicellular organisms. Note, that especially in multicellular organisms, the long-term response of the organism to radiation may even be more important, than radiation damage itself. Cell–cell communication; partly mediated by biological fluids like blood, often involves glycosylation. Bearing these in mind, in the present paper we focus on evaluating long-term glycosylation changes in plasma proteins.

Diversity of proteins and types of molecular pathways they are involved in are enormously expanded due to post-translational modifications. Effects of radiation has already been studied in case of phosphorylation [6,11–13], acetylation [12], ubiquitination [14], carbonylation [15,16], nitrosylation [15,16] and glycosylation [17–20] in different tissues and biofluids and in some cases significant alterations were found.

Glycosylation is one of the most important and most common post-translational modification of proteins. Glycosylation has important biological roles: it takes part in transport of proteins, immune response, communication between cells and several human plasma glycoproteins have been proved to be biomarkers for different diseases [21–24]. From analytical point of view there are two ways (and combination of these) to characterize glycosylation: (1) averaging glycosylation data: before analysis glycans are removed from proteins using specific enzymes (e.g.: *N*-glycans are cleaved with PNGaseF) or acidic hydrolysis, and then released glycan mixture is characterized by the number of antennas and sialic acid residues. In this case important information about attachment sites of glycans is lost. (2) Site-specific glycosylation patterns are carrying more and different biological information: proteins are digested using endoproteases (e.g.: trypsin) and resulted glycopeptides are analyzed, therefore glycosylation sites and site-specific distribution of glycans can also be determined [25–28].

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To our knowledge there are only a few studies published about the relationship between glycosylation and ionizing radiation. (1) Dorsal skin irradiation of *mice* was performed at different doses (20, 40 and 80 Gy) and serum proteins were analyzed using two dimensional differential in-gel electrophoresis. Glycans were analyzed after they were removed from the proteins using mass spectrometry. Shifts in the isoelectric point (*pI*) were observed [17,29] possibly caused by changes in glycosylation, and decrease in biantennary structure, increase in multiantennary *N*-glycans, outer branch fucosylation and sialylation was found [17]. However, because averaging glycosylation was studied, there was no information whether changes in glycosylation involved all, or only some specific proteins. (2) In another study [20] mice were exposed to ionizing radiation (0, 3, 6 and 10 Gy) and plasma glycoproteins were analyzed using lectins. Time and dose dependent alterations of glycoproteins containing galactose, *N*-acetylgalactosamine and mannose were found, however there was no significant difference in the plasma glycoprotein level. (3) In colon cancer cells, correlation between radiation-mediated sialylation of integrin β1 (glycosylated cell surface protein) and increased radio-resistance was observed [18,19].

In the current study *site-specific glycosylation* pattern of plasma proteins was analyzed from *human* samples. Patients suffering from head and neck cancer were treated with radiotherapy (RT). Site-specific glycosylation pattern was determined from samples collected before, during and after the treatment. We observed that glycosylation of various proteins changed significantly, which may help to better understand the molecular response of the human body for radiation.

2. Materials and methods

2.1. Samples and chemicals

Ten patients (Caucasians; 50 to 80-year-old, median 61 years; 7 men) with head and neck squamous cell carcinoma (HNSCC) located in larynx (7) or pharynx (3) were enrolled into the study. All patients were subjected to intensity-modulated RT (IMRT) using 6-MV photons. Total radiation dose delivered to the gross tumor volume (20–148 cm^3) was in the range of 51–72 Gy (median 67.2 Gy); overall treatment time was 22–50 days (median 39 days) with dose fractions 1.8–3 Gy. Three consecutive blood samples (5 mL each) were collected from each patient: pre-treatment sample A, post-treatment sample B collected in the last day of the treatment (i.e. after receiving total dose) and post-treatment sample C collected 1–1.5 month after the end of RT. Plasma specimen was isolated from EDTA-treated blood after centrifugation at 2000 × *g* for 15 min, and then stored at −80 °C before analysis. The study was approved by the appropriate Ethics Committee and all participants provided informed consent indicating their conscious and voluntary participation.

1,4-Dithio-L,D-threitol (DTT) and 2-iodoacetamide (IAA) were obtained from Fluka Chemie GmbH (Sigma–Aldrich®, Zwijndrecht, Netherlands). RapiGest SF (lyophilized sodium-3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)-methoxyl]-1-propane-sulfonate) was purchased from Waters (Milford, MA, USA). Mass spectrometry grade trypsin was purchased from Promega Corporation (Madison, WI, USA).

All other reagents were purchased from Sigma–Aldrich® (St. Louis, MO, USA).

2.2. Immunoaffinity depletion of plasma samples

To reduce sample complexity, two high abundance proteins: albumin and IgG were removed from human plasma with Agilent Multiple Affinity Removal Spin Cartridge HSA/IgG (Agilent Technologies, Santa Clara, CA, USA).

30 μL plasma sample was loaded onto the cartridge and manufacturer's standard protocol was followed. At the end K_2HPO_4 and citric acid were added to the diluted samples to prevent aggregation (30–30 mM concentration in the samples), and samples were concentrated with 10 kDa centrifuge filters. After filtration, volume of the depleted plasma sample was 25 μL.

2.3. Fractionation of depleted plasma samples

Fractionation of depleted plasma samples was performed using Acquity UPLC® System (Waters, Milford, MA, USA).

Poros R2HPLC column (poly(styrene-divinylbenzene), 10 μm, 2.1 × 100 mm, Applied Biosystems, Foster City, CA, USA) was used, and depleted plasma samples were injected in 15 μL volume. The column temperature was 65 °C, the flow rate was 1 mL/min. The gradient was the following: starting with 20% B for 0.7 min, then a 15 min long gradient from 20% to 70% solvent B, increasing to 95% solvent B in 0.1 min, washing for 1.5 min, returning to 20% B in 0.1 min and equilibration for 6 min. Solvent A was water containing 0.07 v/v% trifluoroacetic acid and solvent B was acetonitrile containing 0.07% v/v trifluoroacetic acid.

500 μL fractions were collected manually from 4.5 to 5.5 min (two half minute fractions). To neutralize TFA and to prevent aggregation 1.3 μL NH_3 solution (25 w/w%) and 1.8 μL K_2HPO_4 solution (500 mM) were added to the fractions, and fractions were concentrated to 30 μL with SpeedVac (miVac Duo Concentrator, Genevac Ltd., Ipswich, Suffolk, UK).

Details of this fractionation method and it's benefits during the analysis of glycoproteins was described before [30].

2.4. In-solution digestion

Concentrated fractions were digested as the following: after adding 5 μL NH_4HCO_3 solution (200 mM) proteins were unfolded and reduced using 3 μL RapiGest SF solution (0.5 w/v%) and 2 μL DTT solution (100 mM) for 30 min at 60 °C. Proteins were alkylated using 4 μL NH_4HCO_3 solution (200 mM) and 2 μL IAA solution (200 mM) for 30 min at room temperature in dark. Digestion was performed by adding 1.5 μL trypsin solution (40 μM) for 180 min at 37 °C. Digestion was stopped by adding 1.5 μL formic acid, followed by 30 min incubation at 37 °C. Samples were centrifuged at 13,500 rpm (corresponding to 17,000 × *g*) for 10 min.

2.5. Nano LC–MS(/MS) analysis

Digested fractions were analyzed using nanoAcquity UPLC (Waters, Milford, MA, USA) coupled to a high resolution QTOF Premier mass spectrometer (Waters, Milford, MA, USA).

The chromatographic conditions were the following: Symmetry C18 trap column (180 μm i.d. × 20 mm, Waters Milford, MA, USA) and reversed-phase analytical column (C18, 1.7 μm BEH particles, 75 μm i.d. × 200 mm, Waters, Milford, MA, USA) were used. Column temperature was 55 °C. Using 250 nL/min flow rate the gradient started from 3% to 8% B in 4 min, followed by a 65 min long gradient going to 40% solvent B. Washing was performed using 450 nL/min flow rate and a 2 min long gradient from 40% to 75% solvent B, keeping here for 18 min. Finally, after returning to 3% B in 2 min, equilibration was done for 18 min. Solvent A was water containing 0.1 v/v% formic acid and solvent B was acetonitrile containing 0.1 v/v% formic acid.

Protein composition of the digested fractions was determined with tandem mass spectrometry using data dependent analysis of the resulted peptides. Conditions were the following: electrospray ionization mode was used, capillary voltage was 2.3 kV, nanoflow

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