Analysis of a lectin microarray identifies altered sialylation of mouse serum glycoproteins induced by whole-body radiation exposure

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ABSTRACT

Microarrays containing 45 different lectins were analyzed to identify global changes in the glycosylation of serum glycoproteins from mice exposed to whole-body γ-radiation. The results showed that radiation exposure increased and decreased the relative amounts of α-2,3- and α-2,6-sialic acids, respectively. The expression of α-2,3- and α-2,6-sialyltransferase genes in the liver was analyzed to determine whether changes in their expression were responsible for the sialic acid changes. The increase in α-2,3-sialic acid correlated with S3galS upregulation after radiation exposure; however, a decrease in St6gal1 expression was not observed. Analysis of a PCR array of genes expressed in irradiated mouse livers revealed that irradiation did not alter the expression of most of the included genes. These results suggest that glycomic screening of serum glycoproteins using lectin microarrays can be a powerful tool for identifying radiation-induced changes in the post-translational addition of sugar moieties to proteins. In addition, the results indicate that altered sialylation of glycoproteins may be an initial response to acute radiation exposure.

Keywords: lectin array; glycosylation; adipsin; sialic acid; sialyltransferase

INTRODUCTION

Exposure to radiation can have a variety of deleterious effects on living organisms, and these effects are dependent on the radiation dose as well as on other factors, including mode of exposure (e.g. acute vs chronic, internal vs external, and intermittent vs continuous) [1]. Therefore, accurate dose estimation is essential in making decisions regarding the course of treatment. In general, estimating the radiation dose using biological materials (i.e. biodosimetry) is applicable for patients who do not routinely carry a personal dosimeter. Evaluation of chromosomal aberrations in peripheral blood lymphocytes is the most reliable method for estimating radiation exposure dose.

Radiation-induced DNA damage may alter gene expression. Recently, radiation exposure was shown to also alter the cellular levels of non-coding RNAs. In addition to inducing quantitative changes in expression, radiation exposure can cause epigenetic, covalent modifications of DNA (e.g. methylation) and/or histones (e.g. acetylation and methylation) and thus has the potential to alter chromatin structure and/or function [2, 3].

Certain proteins undergo post-translational modifications, including disulfide bond formation, glycosylation, phosphorylation and acetylation, and these modifications can be altered by radiation [4–6]. Protein glycosylation is an important post-translational modification, and the various glycosylation patterns can greatly affect the function of a protein [7]. Most serum proteins are glycosylated in the liver and then transported to the blood [8].

In previous work, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and electrospray
ionization Fourier transform MS to show that the levels of hepcidin-2, a liver peptide, and histamine and its metabolite 1-methylhistamine in mouse urine were altered after whole-body γ-irradiation [9, 10]. The amounts of these molecules in the urine increased after radiation exposure, supposedly via alterations in iron metabolism, the immune response, and inflammation. In addition, we recently reported that irradiation with 0.25 Gy of γ-rays deglycosylated mouse urine adipsin without affecting its expression according to peptide-mass fingerprinting in combination with MALDI-TOF-MS [11, 12]. Adipsin, also known as complement factor D, is a serine protease that exists in two different glycosylated forms in mice: one with a molecular mass of 44 kDa and the second with a mass of 37 kDa (the deglycosylated protein has a molecular mass of ~26 kDa) [13]. Adipsin is a rate-limiting enzyme in the alternative complement-activation pathway. Because human adipsin does not have an N-glycosylation consensus sequence [14], changes in its N-glycosylation state cannot be used as a biomarker to evaluate radiation exposure in humans. The deglycosylation of adipsin upon radiation exposure suggests that changes in the glycosylation pattern of serum proteins might be a biomarker for excessive radiation exposure in humans.

In the present study, we characterized a lectin microarray with 45 different lectins to determine the effect of whole-body γ-irradiation on the levels of sugar moieties on mouse serum glycoproteins. Lectin microarrays are widely used to comprehensively detect glycosylation on the levels of sugar moieties on mouse serum glycoproteins. To analyze glycoproteins using MS or related instrumentation, they must be hydrolyzed to yield glycopeptides or free sugars, whereas lectin microarray analysis requires no processing. Lectin microarrays can be used for high-throughput identification of different post-translational glycomes [18]. Analysis of the serum of mice exposed to whole-body γ-irradiation showed that the amounts of α-2,3- and α-2,6-sialic acid increased and decreased, respectively, in response to irradiation. The γ-irradiation–induced increase in serum α-2,3-sialic acid was correlated with the upregulation of its biosynthetic gene, St3gaL5, which encodes the liver enzyme β-galactoside α-2,3-sialyltransferase; however, the expression of St6gaL1, which encodes β-galactoside α-2,6 sialyltransferase, did not decrease, indicating that the decrease in α-2,6-sialic acid must be related to an as yet uncharacterized mechanism.

MATERIALS AND METHODS

Ethics

All animal experiments were approved by the Institutional Animal Experimentation Committee of Hiroshima University, Hiroshima, Japan (Authorization No. A12–22).

Animal experiments and γ-irradiation

Male B6C3F1 mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan) and acclimated to their housing conditions for at least 1 week before experimentation. Details concerning the animal experiments, including γ-irradiation, have been described previously [9]. After acclimation, mice were exposed to 0.25, 4 or 6 Gy of whole-body γ-irradiation (Gammacell 40 Exactor, Best Theratronics, Ottawa, Canada) at a dose rate of 0.9 Gy/min. At various times after irradiation, blood for serum or plasma samples was collected (~1 ml) directly from the heart of each ether-anesthetized mouse. The blood samples were immediately transferred into 1.5-ml tubes and maintained at room temperature for 1 h. Alternatively, blood was added into tubes containing Na2EDTA (Neotube, NIPRO Corporation, Osaka, Japan). After centrifugation, serum or plasma fractions were collected and stored at −80°C. The liver was excised and cut into small pieces (~1.5 mm squares), frozen in liquid nitrogen, and stored at −80°C.

Lectin microarray analysis

Analysis of changes in the glycosylation pattern of serum glycoproteins (n = 2) was performed by GlycoTechnica Ltd (Yokohama, Japan) using a lectin microarray as described in Hirabayashi et al. [18]. For each experimental and control run, the serum protein concentration was determined (Micro BCA Protein Assay Reagent Kit, Thermo Scientific, #23235), and 1 μg total serum protein was labeled with Cy3 Mono-Reactive dye (#PA23011, GE Healthcare, Indianapolis, IN, USA) for 1 h in the dark. Excess, unreacted Cy3 dye was then removed through a 0.5-ml Zeba Desalt Spin Column (Thermo Scientific, #89882). Labeled protein was reacted with the 45 lectins in a LecChip microarray (GP Bio Sciences Ltd, Osaka, Japan), and the intensity of each spot (labeled protein bound to a specific lectin in the microarray) was measured using GlycoStation Tools (Pro Ver. 1.5; GlycoTechnica) [19]. Each experiment was performed three independent times. The average net intensity of each spot was calculated by subtracting the background value from the signal intensity of the three independently acquired spots before averaging their intensities. The averaged intensity of each unique spot was multiplied by 100 and then normalized to the mean intensity of the 45 unique spots in the array.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting

Procedures for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting of plasma adipsin are described in Iizuka et al. (2010) [20]. In brief, centrifuged plasma samples (n = 2) were mixed with SDS-PAGE sample buffer containing 100 mM dithiothreitol. After separation through an SDS-PAGE gel (12% w/v acrylamide), protein bands were transferred to a polyvinyliden difluoride membrane (Millipore, Burlington, MA, USA), followed by incubation with anti-adipsin antibody (M-120, sc-50419, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubating the membrane with a secondary antibody against rabbit (Santa Cruz Biotechnology), adipsin was visualized by reaction with streptavidin-peroxidase and then with 20% (v/v) 3,3′-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Kyoto, Japan) in 50 mM Tris-HCl, pH 7.6, 0.1% (v/v) H2O2 for 1 h. The membrane was then washed three times with distilled water.

RNA extraction and quantitative reverse transcription of total RNA

The procedure for RNA extraction from liver tissue is described in Iizuka et al. (2016) [9]. Briefly, each frozen liver was homogenized in a Multi-beads Shocker (Yasui Kikai, Osaka, Japan) according to the manufacturer’s instructions. Total liver RNA was extracted using
miRNeasy Mini Kit reagents (Qiagen, Valencia, CA, USA). mRNAs related to protein glycosylation were amplified by quantitative reverse transcription-PCR (qRT-PCR) and subjected to a Glycosylation RT2 Profiler PCR Array (PAMM-046Z, Qiagen) according to the manufacturer's instructions. Total RNA (1 μg), pooled from three mouse liver samples, was reverse transcribed into cDNA using RT2 First Strand Kit reagents (Qiagen). The cDNAs were reacted with SYBR Green (RT2 SYBR Green Master Mix, Qiagen) in a StepOnePlus Real-time PCR System (Thermo Fisher Scientific, San Jose, CA, USA). In addition, qRT-PCR of individual genes was performed using SYBR Green (Thunderbird SYBR qPCR Mix; TOYOBO, Osaka, Japan), as described in Iizuka et al. (2016) [9], in a LightCycler96 Real-time PCR System (Roche diagnostics, Mannheim, Germany) with the primers listed in Table 1. Amplification of the gene encoding glyceraldehyde 3-phosphate dehydrogenase served as the internal standard. Relative gene expression levels were calculated using the 2−ΔΔCT method [21].

Data analysis
Results are presented as the mean ± standard deviation (SD). The statistical significance of differences between groups was assessed with the Student's t-test, and P < 0.05 was accepted as statistically significant.

Table 1. Primer pairs for quantitative reverse transcription–polymerase chain reaction

| Gene name | Protein name | Primer a | Primer b |
|-----------|--------------|----------|----------|
| St3gal1b | ST3 β-galactoside α-2,3-sialytransferase 1 | Forward | GACAGTCCACAACGCTCTGA |
| St3gal2b | ST3 β-galactoside α-2,3-sialytransferase 2 | Forward | CCCATACGAGGAGTCTTCA |
| St3gal3b | ST3 β-galactoside α-2,3-sialytransferase 3 | Forward | CCTGGCTGTCACCTACTTC |
| St3gal4b | ST3 β-galactoside α-2,3-sialytransferase 4 | Forward | ATCCAGGCTGGAGATGTT |
| St3gal5b | ST3 β-galactoside α-2,3-sialytransferase 5 | Forward | GTTGGGTCTGGAGGATGTT |
| St3gal6b | ST3 β-galactoside α-2,3-sialytransferase 6 | Forward | GTTGGGTCTGGAGGATGTT |
| St6gal1b | β-galactoside α-2,6- sialytransferase 1 | Forward | TGGGCTTGGGATGATGTT |
| Gapdh | glyceraldehyde-3-phosphate dehydrogenase | Forward | TGGGCTTGGGATGATGTT |

aSequences are written in the 5’ to 3’ direction.
bThese primer sequences were first reported in Kwon et al., PLoS One 2014 [40].
TJA-I, SNA and SSA, which bind α-2,6-sialic acid, were lower in serum samples from mice exposed to radiation than in those from control/non-irradiated mice. Changes in serum GlcNAc levels associated with the lectins *Datura stramonium* agglutinin (DSA), *Lycopersicon esculentum* lectin (LEL), and *Solanum tuberosum* lectin (STL) were also observed.

Changes in mouse blood sialylation levels after radiation exposure correlate with changes in the expression of mRNAs associated with sialylation in the mouse liver. Most serum glycoproteins are synthesized in the liver [22]. Therefore, the expression of the α-2,3- and α-2,6-sialyltransferase genes, which are major regulators of α-2,3- and α-2,6-sialylation, respectively, in the livers of mice irradiated with 4 Gy of γ-rays for various times was compared with that in controls (Fig. 2). *St3gal1* and *St3gal2*, which encode beta-galactoside alpha-2,3-sialyltransferases 1 and 2, respectively, were significantly downregulated at 8 h after irradiation, whereas *St3gal5* was significantly upregulated at 4 and 8 h after irradiation. Conversely, *St6gal1*, which encodes α-2,6-sialyltransferase, was downregulated by 8 h after irradiation, although the decrease was not significant (*P* = 0.07). Finally, because radiation exposure alters the expression of many genes, and not only that of genes involved in sialic acid metabolism, we analyzed global changes in the expression of genes involved in protein glycosylation. Ten and two genes showed 1.5-and 2-fold changes in expression, respectively, in the livers of mice at 24 h after whole-body irradiation with 4 Gy of γ-rays compared with those of the controls; however, other tested genes from liver tissues of irradiated mice remained unchanged compared with the controls (Table 3 and

![Fig. 1. Representative western blot of adipin from the serum of mice irradiated with (A) 0.25 Gy or (B) 4 Gy of γ-rays. Each lane corresponds to an individual sample (two animals per experimental condition). Serum samples were collected at the indicated times after irradiation. Arrowheads identify deglycosylated adipin.](#)

**Table 2. Comparison of the levels (reported as intensities) of lectins bound to serum glycoproteins in mice at 24 h after irradiation with 6 Gy of γ-rays and with those in untreated controls.**

| Lectin | Control | 24 h after irradiation with 6 Gy of γ-rays | Fold change | *P*-value | Reported specificity |
|--------|---------|------------------------------------------|-------------|-----------|---------------------|
|        | Average | SD | CV | Average | SD | CV |          |           |
| MAL_I  | 39.55   | 2.62 | 0.066 | 53.20 | 0.57 | 0.011 | 1.35 | 0.019      | Siaα2-3Galβ1-4GlcNac |
| DSA    | 366.50  | 34.65 | 0.095 | 412.50 | 12.02 | 0.029 | 1.13 | 0.218      | (GlcNacβ1-4)n, Galβ1-4GlcNac |
| LTL    | 1.11    | 0.08 | 0.076 | 1.24 | 0.11 | 0.086 | 1.12 | 0.323      | Fucα1-6GlcNac, Fucα1-3(Galβ1-4)GlcNac |
| PHA (E)| 214.00  | 7.07 | 0.033 | 237.00 | 19.80 | 0.084 | 1.11 | 0.262      | bi-antennary complex-type N-glycan with outer Gal and bisecting GlcNac |
| LEL    | 389.50  | 27.58 | 0.071 | 415.00 | 7.07 | 0.017 | 1.07 | 0.333      | GlcNac trimers/tetramers |
| STL    | 384.50  | 0.71 | 0.002 | 398.50 | 6.36 | 0.016 | 1.04 | 0.091      | GlcNac oligomers, oligosaccharide containing GlcNac and MurNac |
| Calsepa| 194.00  | 7.07 | 0.036 | 201.50 | 12.02 | 0.060 | 1.04 | 0.526      | Mannose, maltose |
| SSA    | 595.50  | 3.54 | 0.006 | 565.00 | 7.07 | 0.013 | 0.95 | 0.032      | Siaα2-6Gal/GalNAc |
| SNA    | 610.00  | 8.49 | 0.014 | 558.50 | 13.44 | 0.024 | 0.92 | 0.044      | Siaα2-6Gal/GalNAc |
| TJA-I  | 1009.00 | 43.84 | 0.043 | 910.00 | 60.81 | 0.067 | 0.90 | 0.203      | Siaα2-6Gal/GalNAc |

Note. The lectins listed above satisfied a coefficient of variation of <0.1. *Standard deviation. bCoefficient of variation. cRatio of the average intensity values for mouse serum obtained 24 h after 6 Gy of whole-body γ-irradiation relative to that of the corresponding control. *Student’s t-test. *This information was found in the manufacturer’s documentation.
The results were upregulated by ~1.9-fold and ~1.5-fold, respectively. Conversely, the expression of genes related to α-2,8-sialyltransferase (St8sia3, St8sia4 and St8sia6) was not altered by irradiation. These results suggested that the increase in serum α-2,3-sialic acid was correlated with St3gal5 expression in the mouse liver after radiation exposure, whereas the decrease in serum α-2,6-sialic acid was not related to the expression of any of the genes investigated in this study. The results suggest the existence of another mechanism controlling α-2,6-sialic acid levels on mouse serum glycoproteins.

**DISCUSSION**

Few studies have examined the changes in the glycosylation state of glycoproteins induced by radiation exposure. Two reports noted changes in the glycosylation states of serum glycoproteins in cancer patients who underwent radiotherapy [23, 24]. Changes in glycosylation were also reported in glycoproteins from radiation-exposed human umbilical vein endothelial cells [25] and in mouse serum after dorsal skin irradiation [6]. The present study is the first report to investigate global changes in the glycosylated state of serum glycoproteins from whole-body γ-irradiated mice. The doses used in the present study were inconsistent among experiments. Therefore, different doses and time-course analyses are needed to accurately interpret the data regarding the identification of exposure-related biomarkers associated with radiation-induced aberrant glycosylation patterns. Almost identical changes in the deglycosylation of urine adipsin were observed after exposure to 6 Gy of γ-irradiation and after exposure to 0.25, 0.5, 1 or 4 Gy of γ-irradiation (Supplementary Fig. 1), suggesting that this protein modification occurs in a dose-independent manner; this was supported by the results obtained for plasma adipsin (Fig. 1). Both chromosomal aberrations and γH2AX foci in peripheral blood lymphocytes increased linearly with radiation dose. Consistent with this observation, specific cellular responses such as the radioadaptive response, radiation-induced bystander response, low-dose hyper-radiosensitivity, and genomic instability are dose-dependent responses that involve non-target molecules or molecules that are insensitive to radiation dose. This non-target effect suggests the existence of a minimum effective dose, above which there is little additional effect [26]. Further intensive investigation is required in

![Fig. 2](image-url)  
*Expression of genes associated with sialyltransferases in the mouse liver (four animals per experimental condition) after whole-body irradiation. St3gal1, St3gal2, St3gal3, St3gal4, St3gal5, St3gal6 and St6gal1 expression was analyzed by quantitative reverse transcription–polymerase chain reaction. The relative expression of each gene was compared with the corresponding level in control mice. *P < 0.05; **P < 0.01.*

**Table 3. Ten genes showed at least a 1.5-fold change in expression according to RT² Profiler PCR Array analysis between control values and the values at 24 h after 4 Gy of mouse whole-body irradiation**

| Gene                | Ct value<sup>a</sup> | Fold change<sup>b</sup> | Gene name                                                                 |
|---------------------|----------------------|--------------------------|---------------------------------------------------------------------------|
| St3gal2             | 31.34                | 1.92                     | ST3 β-galactoside α-2,3-sialyltransferase 2                                 |
| Pont2               | 24.54                | 1.66                     | Protein-O-mannosyltransferase 2                                            |
| Galnt7              | 27.58                | 1.64                     | UDP-N-acetyl-α-β-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 |
| B3gnt3              | 27.85                | 1.55                     | UDP-GlcNAc: β-Gal β-1,3-N-acetylgalactosaminyltransferase 3                |
| St6gal1             | 23.32                | 1.54                     | β-galactoside α-2,6 sialyltransferase 1                                    |
| Mgat5               | 26.61                | 1.53                     | Mannoside acetylgalactosaminyltransferase 5                                |
| Galnt14             | 30.63                | 0.65                     | UDP-N-acetyl-α-n-galactosamine:polypeptide N-acetylgalactosaminyltransferase 14 |
| Wbscr17             | 32.52                | 0.53                     | Williams–Beuren syndrome chromosome region 17 homolog (human)             |
| Mgat3               | 32.30                | 0.38                     | Mannoside acetylgalactosaminyltransferase 3                                |
| Galnt5              | 32.81                | 0.34                     | UDP-N-acetyl-α-β-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 |

<sup>a</sup>Threshold cycle value. <sup>b</sup>Ratio of the relative expression value (2<sup>-ΔΔCt</sup>) of ‘24 h after 4 Gy irradiation’ to that of ‘control’.
Radiation expression of specific genes may provide useful evidence for biodosimetry. In a study comparing the expression of 84 liver genes from mice irradiated with 4 Gy of γ-rays with that in the corresponding controls, solid triangles identify genes with at least a 1.5-fold change in expression after irradiation. The central line indicates unchanged gene expression, and boundaries represent the 1.5-fold cut-off value for the change in expression.

In order to validate the observed changes in glycosylation as de facto biomarkers of radiation exposure, exposure to 0.25 Gy of γ-irradiation, but not to 0.05 or 0.1 Gy, resulted in the accumulation of the deglycosylated form of urine adipin (data not shown). Therefore, the deglycosylation of this protein cannot be used as a marker to evaluate the effect of radiation exposure. However, the deglycosylation of this protein has not been reported previously. Radiation exposure induces alterations in the levels of acute-phase proteins, which herald induction of the acute-phase reaction [30]. The levels of α-2,3-, α-2,6- and α-2,8-sialylated serum glycoproteins increase in response to inflammation induced by turpentine oil in mice, and these proteins include hemopexin, haptoglobin β and α-1-acid glycoprotein, which are acute-phase proteins [31]. Moreover, liver St3gal1, St3gal3, St6gal1 and St6GalNAc VI, which are sialyltransferases, are upregulated, whereas St8Sia expression remains unchanged after turpentine oil treatment [31]. Conversely, zymosan-induced peritonitis increases α-2,3-sialylation but not α-2,6-sialylation [32]. Because radiation exposure and inflammation induce the acute-phase response, these results suggest that changes in glycosylation can be used to differentiate between radiation-induced alterations and the inflammatory response in damaged tissue; however, further investigation is required to support this idea.

The changes in protein sialylation after radiation exposure were at least partially related to the expression of the liver glycosyltransferase gene St3gal5. Chaze et al. showed that radiation-induced changes in glycosylation, including changes related to multi-antennary N-glycans and outer-branch fucosylations and sialylations, are associated with the upregulation of genes encoding proteins responsible for those glycosylations [6]. However, GlcNAc-containing glycan chains can be cleaved by hydrogen peroxide in the presence of copper ions in vitro [33], suggesting the existence of a non-enzymatic N-glycan cleavage machinery. Platelets also have glycosyltransferase activity, suggesting that a platelet glycosyltransferase(s) is partially responsible for serum-related glycosylation changes [34].

We identified sialylation changes in mouse serum related to radiation-induced alterations. Most serum glycoproteins are sialylated, as glycoprotein sialylation is a marker for protein disassembly [35]. Sialylated proteins are often found on the cell surface, and these proteins are important for cell–cell interactions, cell migration, cell adhesion, cancer metastasis, and pathogen infection [36]. In addition, the undifferentiated state of bone marrow hematopoietic stem cells is maintained by sialyltransferase activity, including that of extracellular ST6Gal-1 [37]. We also observed the upregulation of St3gal5 in the mouse liver after radiation exposure. St3gal5 encodes an enzyme involved in the synthesis of the ganglioside GM3. Ganglioside GM3 is involved in signal transduction (including insulin signaling), cell differentiation, and cell migration. The presence of ganglioside GM3 suppresses inflammation [38, 39], suggesting that radiation exposure may cause inflammation through the acute-phase reaction. The biological significance of altered α-2,3- and α-2,6-sialylation after radiation exposure and the upregulation of St3gal5 remains unclear, and further studies are needed.

In conclusion, the present observations suggest that the glycosylation states of glycoproteins, especially those involving sialylation, can be used to monitor the effects of radiation exposure, although further investigation is needed to develop new biodosimetry methods specific for radiation exposure.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest associated with this manuscript.

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SUPPLEMENTARY DATA
Supplementary data are available at Journal of Radiation Research online.

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