Sulfation of keratan sulfate proteoglycan reduces radiation-induced apoptosis in human Burkitt’s lymphoma cell lines

Fumiaki Nakayama a,*, Sachiko Umeda a, Tomomi Ichimiya b, Shin Kamiyama b, Masaharu Hazawa c, Takeshi Yasuda c, Shoko Nishihara b, Takashi Imai a

aAdvanced Radiation Biology Research Program, Research Center for Charged Particle Therapy, National Institute of Radiological Sciences, Chiba, Japan
bLaboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University, Hachioji, Japan
cDepartment of Radiation Emergency Medicine, Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences, Chiba, Japan

A R T I C L E   I N F O

Article history:
Received 14 August 2012
Revised 18 October 2012
Accepted 3 December 2012
Available online 10 December 2012

Edited by Vladimir Skulachev

Keywords:
Apoptosis
Burkitt’s lymphoma
Keratan sulfate
PAPST
Radiation

A B S T R A C T

This study focuses on clarifying the contribution of sulfation to radiation-induced apoptosis in human Burkitt’s lymphoma cell lines, using 3-phosphoadenosine 5-phosphosulfate transporters (PAPSTs). Overexpression of PAPST1 or PAPST2 reduced radiation-induced apoptosis in Namalwa cells, whereas the repression of PAPST1 expression enhanced apoptosis. Inhibition of PAPST slightly decreased keratan sulfate (KS) expression, so that depletion of KS significantly increased radiation-induced apoptosis. In addition, the repression of all three N-acetylgalactosamine-6-O-sulfotransferases (CHST2, CHST6, and CHST7) increased apoptosis. In contrast, PAPST1 expression promoted the phosphorylation of p38 MAPK and Akt in irradiated Namalwa cells. These findings suggest that 6-O-sulfation of GlcNAc residues in KS reduces radiation-induced apoptosis of human Burkitt’s lymphoma cells.

© 2012 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Sulfation of a variety of molecules, including glycoproteins, proteoglycans (PGs), and glycolipids, is an important posttranslational modification, which modifies the properties of molecules by conferring a negative charge. Heparan sulfate (HS) and chondroitin sulfate (CS) have highly-sulfated glycosaminoglycan (GAG) chains, which play important roles in many biological processes. For example, a number of fibroblast growth factors (FGFs) interact with these sulfated GAG subtypes [1], so that inhibition of sulfation was able to reduce the signaling of FGF. Therefore, the sulfation of PGs may regulate numerous physiological and pathological events.

Abbreviations: CS, chondroitin sulfate; FGF, fibroblast growth factor; GAG, glycosaminoglycan; Gal, galactose; GlcNAc, N-acetylgalactosamine; GlcNAcST, N-acetylgalactosamine-6-O-sulfotransferase; HS, heparin sulfate; KS, keratan sulfate; MAPK, mitogen-activated protein kinase; PAPST, 3-phosphoadenosine 5-phosphosulfate transporter; PG, proteoglycan

* Corresponding author. Address: Advanced Radiation Biology Research Program, Research Center for Charged Particle Therapy, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan. Fax: +81 43 206 6267.
E-mail address: f_naka@nirs.go.jp (F. Nakayama).

The process of sulfation requires the involvement of a high energy form of the universal sulfate donor, namely, 3-phosphoadenosine 5-phosphosulfate (PAPS). In higher organisms, PAPS is synthesized in the cytosol or nucleus by PAPS synthetases [2], and PAPS transporters (PAPSTs) transfer PAPS from the cytosol into the Golgi lumen for the sulfation of sugar residues. Recently, two members of the PAPSTs, PAPST1 (SLC35B2) and PAPST2 (SLC35B3), were identified in humans [3,4] and it was suggested that PAPSTs regulate the sulfation process in addition to sulfotransferases [5].

It has been reported that some sulfated structures alter the expression of molecules associated with the progression of cancer. In colon adenocarcinoma, the amount of sulfated glycoproteins decreased in the order normal mucosa, primary tumors, and metastatic tumors [6], and down-regulation of galactose-3-O-sulfotransferase-2 reduced the expression of sulfomucins in colon adenocarcinomas [7]. Instead, an increase in sialylation was associated with malignant transformation of colonic epithelial cells. The change of mucin production from sulfated to sialylated is a significant phenotypic alteration during tumor progression.

This study investigated the contribution of sulfation of PGs to radiation-induced apoptosis using PAPSTs and identified that the sulfation of keratan sulfate proteoglycan (KSPG) caused radioresistance in human Burkitt’s lymphoma cell lines.
2. Materials and methods

2.1. Cell culture and reagents

Human Burkitt’s lymphoma cell lines Raji and Daudi were obtained from RIKEN Bio Resource Center and Namalwa was obtained from ATCC, and they were maintained in a medium consisting of RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS). Anti-HS (HepSS-1, Invitrogen), anti-CS-A (2H6), anti-CS-D (MO-225), and anti-keratan sulfated (KS) (5-D-4, BCD-4) monoclonal antibodies were purchased from Siekagaku Kogyo (Tokyo, Japan).

2.2. Transfection of PAPST genes

Namalwa cells were transfected with pAMo vectors [8] containing human PAPST1 or PAPST2 genes by electroporation using a GenePulser apparatus (Bio-Rad, Richmond, CA, USA) (Namalwa-PAPST1, or Namalwa-PAPST2). The transfectants were selected by the addition of 0.6 mg/ml geneticin (G418) (Gibco) to the medium.

2.3. In vitro siRNA assay

Stealth RNAi is a type of chemically modified siRNA obtained from Invitrogen (Carlsbad, CA, USA). The synthesized oligonucleotides for the target gene of each were as follows: PAPST1, 5'-ugg acc cag cua ugg uuc ucg cga u-3'; the negative control for PAPST1, 5'-ugg acc ugc gua uug uuc ucg cga u-3'; PAPST2, 5'-aau ccc gaa aac aaa ugg uuc ugc uca u-3' and 5'-aau ugc ugc ccc uaa ugg uuc ugc cga cca gga agu u-3'; CHST-2, 5'-ccc cuu gga aac aag ugg uuc ugc cga u-3' and 5'-aau ugc ugc uca ucc uca ucg cca gga agu u-3'; CHST-7, 5'-aau ugc ugc uca ucc uca ucg cca gga agu u-3'; and CHST-8, 5'-aau ugc ugc uca ucc uca ucg cca gga agu u-3'. Each Stealth RNAi duplex was transfected at a final concentration of 50 nM using Lipofectamine™ RNAiMAX in accordance with the manufacturer's protocol (Invitrogen).

2.4. Quantitative RT-PCR assay

A quantitative RT-PCR assay was performed to determine the amount of each transcript in Namalwa cells using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The absolute amount of each transcript (copies/μl) was divided by that of β-actin (copies/μl) for normalization. Each normalized amount was further divided by that of the control sample to obtain the relative rate of expression.

2.5. Antibody array

The protein expression profile of Namalwa cells was determined using a Human Phospho-MAPK Array Kit in accordance with the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA). Briefly, Namalwa-pAMo cells transfected with stealth RNAi targeting the PAPST1 gene and Namalwa-PAPST1 cells transfected with the negative control stealth RNAi were irradiated with 20 Gy of X-rays 48 h after transfection. The cell lysates were prepared 6 h after irradiation and each array was incubated with 200–300 μg of lysate protein. The signals were generated using ECL Plus Western Blot Detection Reagents (GE Healthcare, Little Chalfont, UK) and detected using a luminescent image analyzer LAS-4000 mini (Fujifilm, Tokyo, Japan). The detected signals were quantified using Multi Gauge ver 3.0 image analysis software (Fujifilm). Each value was calculated as a relative value compared with that in the positive control.

2.6. Western blot analysis

Phosphorylation of p38 and Akt was also examined by western blot analysis. A 50 μg sample of each lysate of transfecteds was electrophoresed on an any KD Mini-PROTEAN TGX gel (BioRad) and transferred to an Immobilon PVDF membrane. Anti-phospho-p38 MAPK (Thr180/Tyr182) (9211) and anti-p38 MAPK (9212) antibodies (Cell Signaling Technology, Danvers, MA, USA) were used as probes at 1:500 dilution. An anti-β-Actin antibody (4967) was used as loading control at 1:1000 dilution (Cell Signaling Technology).

2.7. Apoptosis detection

Radiation-induced apoptosis was assessed by microscopic examination of nuclear morphology using Hoechst 33258 (Calbiochem, La Jolla, CA, USA), as described previously [9]. Briefly, cells were plated at a density of 3 × 10⁶ cells per 3.5-cm dish and irradiated with X-rays. The condensed chromatin of nuclei was visualized by staining with 0.1 mg/ml of Hoechst 33258 after fixation with glutaraldehyde. The percentage of apoptotic cells was determined from the examination of 2,000 cells in 10 fields.

3. Results

3.1. The overexpression of PAPST in Namalwa cells decreases radiation-induced apoptosis

We evaluated the transcript levels of PAPST1 and PAPST2 in a human B-lymphoma cell line, Namalwa cells (Fig. 1A). The level of PAPST1 transcripts was approximately 5-fold higher than that of PAPST2. Namalwa cells were transfected with an expression vector containing the coding sequence of human PAPST1 or PAPST2. The level of PAPST1 transcripts in Namalwa-PAPST1 cells was 2.5-fold higher than that in Namalwa-pAMo or Namalwa-PAPST2 cells. In contrast, the level of PAPST2 transcripts in Namalwa-PAPST2 cells was approximately 3-fold higher than that in Namalwa-pAMo or Namalwa-PAPST1 cells and reached 80% of the level of PAPST1 transcripts in Namalwa-pAMo cells.

The level of apoptosis was determined in each transfecant by Hoechst staining 24 h after irradiation (Fig. 1B). Irradiation increased the apoptosis of Namalwa-pAMo cells in a dose-dependent manner. In contrast, overexpression of PAPST decreased radiation-induced apoptosis in Namalwa cells. In particular, the most significant inhibition of apoptosis was seen at 20 Gy of irradiation, and the number of apoptotic cells in Namalwa-PAPST1 and -PAPST2 cells was approximately 60% of that in Namalwa-pAMo cells. Overexpression of PAPST1 and PAPST2 decreased apoptosis by almost the same extent.

3.2. Repression of PAPST increases radiation-induced apoptosis in Namalwa cells overexpressing each PAPST

Namalwa-PAPST1 or -PAPST2 cells were transfected with stealth RNAi targeted against the PAPST1 or PAPST2 gene, respec-
tively (Fig. 1CD). Each Namalwa transfectant was irradiated with X-rays at 48 h after transfection, and then apoptosis of the cells was determined by Hoechst staining. Repression of each PAPST in Namalwa cells overexpressing the corresponding PAPST gene significantly increased the population of apoptotic cells at 20 or 30 Gy of irradiation.

3.3. KSPG is expressed in Namalwa cells

The level of cell surface expression of proteoglycans was studied by flow cytometry using anti-HS, anti-CS (CS-A, CS-D), and anti-KS antibodies. HS was not expressed on the surface of Namalwa cells and the expression levels of CS-A and CS-D were extremely low, whereas KS was significantly detected using the anti-KS antibody (BCD-4) (Fig. 2A). However, Namalwa cells were not positively stained with another anti-KS antibody (5-D-4), which was highly specific for sulfated poly(N-acetyllactosamine) domains of KS chains. Inhibition of sulfation using chlorate, an inhibitor of PAPS sulfurylase, slightly reduced the expression level of the BCD-4 epitope in Namalwa cells (Fig. 2B). In addition, siRNA-mediated PAPST1 repression decreased the level of the BCD-4 epitope, although the repression of PAPST2 did not reduce it (Fig. 2C). Namalwa cells repressing both PAPSTs reduced the level of BCD-4 epitope to the same extent as cells repressing only PAPST1.

3.4. KSPG decreases radiation-induced apoptosis in Namalwa cells

Namalwa, Daudi, and Raji cells were incubated with keratanase for eliminative cleavage of KS chains from the cell surface. The level of KSPG expression was determined in the treated cells by flow cytometric analysis using the BCD-4 antibody, and it was confirmed that KSPG was completely removed from these cells (Fig. 3A). In addition, the absence of KS chains on the cell surface significantly enhanced radiation-induced apoptosis in Namalwa cells at all the doses tested (Fig. 3B). In contrast, heparitinase I or chondroitinase ABC treatment did not significantly increase radiation-induced apoptosis in Namalwa cells; however, these treatments tended to enhance it (data not shown). Hence, the expression levels of HS and CS in Namalwa cells might be too low to affect apoptosis.

3.5. Sulfation of KSPG plays a significant role in the inhibition of radiation-induced apoptosis in Namalwa cells

The sulfation process enhanced by PAPST1 may be involved in the inhibition of radiation-induced apoptosis in Burkitt’s lymphoma cell lines. Two galactose-6-O-sulfotransferases (Gal6ST) (CHST1, CHST3) and three N-acetylgalcosamine-6-O-sulfotransferases (GlcNAc6ST) (CHST2, CHST6, and CHST7) were identified as enzymes involved in the sulfation of KSPG [10–12] (Fig. 4A). However, only three sulfotransferases, CHST2, CHST6, and CHST7 were expressed in Namalwa cells (data not shown). Therefore, each gene was repressed in Namalwa cells using single siRNA transfection to determine which sulfation is involved in radiation-induced apoptosis. However, there was no significant difference in apoptosis among transfectants after irradiation at 10, 20, or 30 Gy (data not shown). Moreover, double siRNA transfection did not enhance radiation-induced apoptosis except for the repression of both CHST6 and CHST7 (Fig. 4B). In contrast, the triple repression of CHST2, CHST6, and CHST7 reduced each transcript level by 44%, 68%, and 32%, respectively, (Fig. 4C), and it significantly increased radiation-induced apoptosis (Fig. 4D). These findings suggest that sulfation of KS is essential for promoting anti-apoptotic signaling after irradiation; in particular, the KS-mediated apoptosis depends on 6-O-sulfation of GlcNAc residues.

3.6. Expression of PAPST1 is involved in the activation of Akt and p38 MAPK in Namalwa cells

The relative levels of phosphorylation of nine mitogen-activated protein kinases (MAPKs) and nine other serine/threonine kinases were analyzed using an antibody-based array to understand the
roles of PAPST1 in mechanisms underlying its anti-apoptotic effects (Fig. 5A). Phosphorylation of p38 MAPK in PAPST1-overexpressing cells increased more than twofold that in PAPST1-repressed cells after irradiation (Fig. 5B). Phosphorylation of Erk and Akt increased slightly in PAPST1-overexpressing cells compared with PAPST1-repressed cells, although these cells showed the same level of p38 protein expression (Fig. 5C).

The inhibition of PI-3K, MEK, or p38 MAPK in Namalwa cells was performed using appropriate inhibitors (LY294002, PD98059, and SB203580, respectively) (Fig. 5D). Inhibition of PI-3K or p38 MAPK in Namalwa cells increased radiation-induced apoptosis, whereas inhibition of MEK did not increase radiation-induced apoptosis.

4. Discussion

KS is expressed in the cornea, cartilage, bone, brain, and epithelial tissues, but it was not reported whether lymphoma cells expressed KS on the cell surface. However, it seems that KS molecules modify a variety of proteins with a range of localization patterns [13]. In this study, two kinds of anti-KS antibodies (5-D-4 and BCD-4) were used for the analysis of the cell surface expression of KS. 5-D-4 is highly specific for sulfated poly(N-acetylactosamine) domains on KS oligosaccharides [14], whereas BCD-4 reacts with KS bound to a core protein [15]. Burkitt’s lymphoma cell lines were stained with BCD-4 but not 5-D-4 (Fig. 3A), so they were likely to express sparsely-sulfated KS. In addition, repression of PAPST, which reduced sulfation, slightly decreased the expression of the BCD-4 epitope in Namalwa cells (Fig. 2B), whereas the BCD-4 epitope completely disappeared after keratanase treatment (Fig. 3A). Although Daudi and Raji cells expressed a low level of the BCD-4 epitope, keratanase treatment significantly increased apoptosis in these cells (Fig. 3B). Thus, these findings suggest that KS plays an essential role in apoptosis in Burkitt’s lymphoma cell lines.

PAPST1 translocates PAPS from the cytosol into the Golgi lumen in Namalwa cells, and then sulfate is transferred from PAPS to position 6 of GlcNAc on the KS chains by three sulfotransferases (CHST7, CHST2, and CHST6). Sulfation of KS chains was essential for anti-apoptotic signaling, and the level of PAPST1 expression was correlated with the extent of apoptosis induced by X-rays. In contrast, the level of the PAPST2 transcript was less than one-fourth of that of PAPST1, so that PAPST2 had less influence on the level of the BCD-4 epitope (Fig. 2C), and siRNA-mediated repression of PAPST2 did not increase radiation-induced apoptosis (Fig. S1). However, PAPST2 has the potential to play a similar role to PAPST1 because overexpression of PAPST2 reduced radiation-induced apoptosis (Fig. 1B).

KSPG is important for the transparency of corneal tissue; however, the functional properties of KS such as anti-apoptotic effects in other tissues are still unknown. In this study, the expression of PAPST1 reduced radiation-induced apoptosis in Namalwa cells by activation of the p38 MAPK and PI-3K pathways through KS biosynthesis (Fig. 6). In addition, pro-apoptotic signaling molecules increased in PAPST1-repressed cells (Fig. S2). The anti-apoptotic role of the PI-3K/AKT pathway has been well documented, and the p38 pathway played an important role in radioprotection through radiation-induced G2/M arrest in some cell types [16].

Various sulfated GAG subtypes can interact with FGFs [1], which strongly enhances radioprotection including anti-apoptotic effects [17]. Hematopoietic cells rarely express FGF receptors, and phosphorylation of FGF receptor tyrosine kinases was not detected in Namalwa-PAPST1 cells after irradiation by an antibody-based array (data not shown); however, FGFs might inhibit...
Fig. 3. Inhibitory effects of KSPG on radiation-induced apoptosis in Burkitt’s lymphoma cell lines. Three Burkitt’s lymphoma cell lines were incubated with 100 mU/ml keratanase for 60 min to eliminate KSPG on the cell surface. (A) The treated cells were stained with anti-KS antibody (BCD-4) and subjected to FACS. (B) Cells were irradiated with X-rays at a dose of 10, 20, or 30 Gy after keratanase treatment. The percentage of apoptotic cells was determined by Hoechst 33258 staining. Values represent the mean ± S.D. Similar findings were observed in three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 4. Involvement of three sulfotransferases in anti-apoptotic effects in Namalwa cells. (A) Among five sulfotransferases involved in KS synthesis, CHST2, CHST6, and CHST7 could be detected in Namalwa cells but CHST1 and CHST3 could not. (B) Two of three sulfotransferases were repressed in Namalwa cells after double siRNA transfection, and apoptosis was evaluated 24 h after irradiation. (C) All three sulfotransferases were inhibited with the triple siRNA transfection. Relative levels of CHST7, CHST2, and CHST6 transcripts in the transfectants were determined by quantitative RT-PCR. (D) The rate of apoptotic cells was determined in triple sulfotransferase-repressed cells 24 h after irradiation. Values represent the mean ± S.D. Similar findings were observed in three independent experiments. *P < 0.05; **P < 0.01.
radiation-induced apoptosis without any involvement of FGF receptors because FGFR2 can be internalized into cells to play a role in physical events [18]. Therefore, unknown extrinsic factors might be involved in the activation of the p38 MAPK and PI-3K signaling pathways through the mediation of KS, resulting in activation of an anti-apoptotic pathway in Namalwa cells (Fig. 6).

Burkitt’s lymphoma is a highly aggressive B-cell non-Hodgkin lymphoma [19]. Intensive chemotherapy is the standard therapy of Burkitt’s lymphoma, whereas radiation therapy is not a component of treatment because it is not effective for the local control of Burkitt’s lymphoma [20]. Outcomes with intensive chemotherapy are now excellent in children owing to good supportive care, but prognosis is still poor in elderly adults [19]. Therefore, a reduction in the toxic effects of treatment is needed in order to improve outcomes and the specific enhancement of radiosensitivity of Burkitt’s lymphoma is useful for developing treatments.

This study concluded that the sulfation of KS represents a potential target for radiotherapy of Burkitt’s lymphoma.

Acknowledgements
This work was supported by KAKENHI (24591856).

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.12.002.

References


