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Nm23-H1 is responsible for SUMO-2-involved DNA synthesis induction after X-ray irradiation in human cells

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ABSTRACT

Human cells derived from nevoid basal carcinoma syndrome (NBCCS) patients show increased levels of DNA synthesis activity after X-ray irradiation which is suggested to be casually related to reduction in cellular amounts of small ubiquitin-like protein modifier (SUMO-2/SMT-3A). In the present study, an increased level of DNA synthesis activity was found 8 h after X-ray irradiation in HeLa cells with reduction in SUMO-2 amounts by siRNA treatment for *SUMO-2*. When comparative proteomic analysis was performed between the siRNA and mimic control siRNA treated cells using two-dimensional (2D) electrophoresis and mass spectrometry, three proteins were identified as candidates. Our research focused on Nm23-H1, a nucleoside diphosphate kinase, whose amounts decreased after X-ray irradiation in HeLa cells treated with siRNA for *SUMO-2*. In the Nm23-H1 siRNA treated cells, induction of DNA synthesis was also detected. Furthermore, in synchronized HeLa cells, DNA synthesis was confirmed in the S phase. Moreover, increased expression of proliferating cell nuclear antigen (PCNA) was observed in Nm23-H1 siRNA treated HeLa cells after X-ray irradiation. In addition, Nm23-H1 was modified with SUMO-2 after X-ray irradiation. The present findings suggest that the reduction of Nm23-H1 is related to the decrease in sumoylation, which in turn, is involved in the induction of DNA synthesis via the regulation of PCNA expression after X-ray irradiation.

Introduction

As a ubiquitous phenomenon, there is no increase of DNA synthesis activity levels in human cells exposed to ionizing radiation such as X-rays. However, we found that the level of DNA synthesis activity is unusually increased in X-ray-irradiated NBCCS cells, derived from patients with Gorlin syndrome, an autosomal dominant disorder that results in a predisposition to developmental defects and cancer [1]. Moreover, we found that induction of DNA synthesis after X-ray irradiation is associated with reduction in cellular amounts of SUMO-2/SMT3A in NBCCS cells [2]. However, the molecular mechanisms which are responsible for the decreased levels of SUMO-2 expression leading to the induction of DNA synthesis in NBCCS cells are still unclear.

To date, four mammalian SUMO proteins have been identified, including SUMO-1/SMT3C, SUMO-2/SMT3A, SUMO-3/SMT3B and SUMO-4. Human SUMO-2/SMT3A was discovered by cDNA selection directly from the telomeric region of chromosome 21q that is associated with Down syndrome [3]. Under various kinds of genotoxic stresses including X-ray irradiation, an increase in the amounts of SUMO-2, which are incorporated into high molecular weight complexes within cells, has been reported [4]. Azuma et al. reported SUMO-2 modification of topoisomerase II, which may play crucial roles in both chromosome condensation and segregation during mitosis [5]. Other reports have revealed that cell cycle factors, such as PCNA, p53 and pRB, which are involved in DNA replication and repair, can be sumoylated by SUMO-2, and such modification may play roles in stress response [6–8]. Thus it is important to clarify the role of SUMO-2 in DNA synthesis induction.

Further clarification of the molecular mechanism of DNA synthesis induction is challenging. The capacity of proliferation of NBCCS cells is very low, and therefore we need to establish another cell line which grows actively and are suitable for experimentation. Therefore, we here attempted to establish cells in which DNA synthesis levels are induced after X-ray irradiation. As a result, in HeLa cells with a knockdown of SUMO-2 by its specific siRNA, we successfully engineered a model system to study the relationship between the down-regulation of SUMO-2 and the induction of DNA synthesis after X-ray irradiation. Using this system, we then performed proteomic analysis using 2D electrophoresis in order to

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search for SUMO-2-interacted proteins which are involved in the DNA synthesis induction after X-ray irradiation in the siRNA treated HeLa cells.

Materials and methods

Cells and culture conditions

The cervical tumor cell line, HeLa S3, was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). Cells were cultured in Eagle's minimum essential medium (EMEM) (Nissui, Tokyo, Japan) containing 10% (v/v) calf serum (Invitrogen, CA, USA) at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2.

X-ray irradiation

X-ray irradiation was performed as described previously [2]. In brief, an X-ray irradiation apparatus (MBR-1505R, Hitachi Medical Corp., Tokyo, Japan) was operated at 150 kV and 20 mA with a 0.5 mm aluminum plus 0.1 mm copper filter and at a dose rate of 2.0 Gy/min.

Analysis of DNA synthesis activity

The cellular DNA synthesis activity was measured using a pulse-labeling method as previously described [2]. Briefly, cells were cultured in medium containing 0.06 mCi/mL [2-^{14}C]deoxythymidine (dThd) (53 mCi/mmol; PerkinElmer, Boston, USA) for 24 h. A total of 20 h after seeding, 10^5 cells per dish were irradiated with X-rays, and then pulse-labeled by culture with EMEM containing 50 μCi/mL [methyl-^{3}H]dThd (75 Ci/mmol; PerkinElmer) for 10 min, followed by treatment with trichloroacetic acid to insoluble cell materials. Then, the cellular components were harvested using a Harvester 96 (TOMTEC, Hamden, USA), and the radioactivity was counted by using a 1450 Microbeta liquid scintillation counter (Wallac, Turku, Finland). Levels of cellular DNA synthesis activity were calculated as the ^{3}H/^{14}C radioactivity ratios.

Analysis of DNA synthesis activity in synchronized cells

Cells were cultured in medium containing 0.06 mCi/mL [2-^{14}C]dThd for 24 h followed by the synchronization of cells at the G1/S boundary phase which was carried out by two cycles of thymidine block, according to the method described previously [9]. Briefly, the cells were successively exposed to 2.6 mM thymidine for 16 h, to normal medium for 8 h, and to 2.6 mM thymidine for 16 h. After being released from the second thymidine block, the cells progressed through S phase and into the following phases. Cells were pulse-labeled by culture with EMEM containing 50 μCi/mL [methyl-^{3}H]dThd for 10 min, followed by treatment with trichloroacetic acid to precipitate cell materials. DNA synthesis activity rates were calculated as the ^{3}H/^{14}C radioactivity ratios. Cell number was periodically counted with a hemocytometer.

Measurements of cell proliferation rate

The cell proliferation rate was measured as described previously [10]. Briefly, cells were seeded in a 35-mm dish (8×10^4 cells/dish) and cultured for 5 days. The total number of cells per dish was counted daily with a hemocytometer. Numbers of spontaneously dead cells at the stationary growing stage were counted using trypan-blue staining.

Two-dimensional electrophoresis

Proteins obtained from cell lysates were desalted prior to being analyzed by two-dimensional differential gel electrophoresis (2D-PAGE)[1] as described previously [11,12]. Briefly, the first dimension was performed on a separation from a 7 cm immobilized PH gradient (IPG) immobiline dry strips with a pH range between 3 and 10 (Amersham Biosciences, Uppsala, Sweden) using an IPGPhor electrophoresis unit (Amersham Biosciences, Uppsala, Sweden). Lyophilized protein samples were suspended in an isoelectric focusing (IEF) sample buffer containing 8 M urea, 65 mM dithiothreitol (DTT), 2% 3-[(3-choloamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) and 0.5% IPG buffer (Amersham Biosciences, Uppsala, Sweden). Approximately 100 μg of protein in 350 μL of isoelectric focusing sample buffer was loaded on each strip. The IPG immobiline dry strips were actively rehydrated with the samples at room temperature for 20 h in the ceramic strip holders. The separation was carried out with a voltage gradient of 250 V for 1 h, from 250 to 1000 V for 1 h, from 1000 to 8000 V for 3.75 h. After IEF gel electrophoresis, strips were rinsed for 15 min with a 50 mM Tris–HCl buffer (pH 6.8), containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS) and 1% (w/v) DTT. Subsequently, the IPG immobiline dry strips were applied to a 8–12% (w/v) polyacrylamide gel gradient, and two-dimensional SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed. Then, the gel was stained by silver staining using a silver staining kit (Invitrogen).

Identification of proteins by mass spectrometry

Protein samples were obtained by cutting spots from the silver-stained gels, followed by silver destaining with silver staining kit (Invitrogen) and identified by mass spectrometry as described elsewhere [13]. Briefly, in-gel tryptic digestion of proteins was performed as described [14]. Digested peptides equivalent to a maximum of 5–10 pmol of a protein were injected into a MAGIC C18 column (AMR, Tokyo, Japan) attached to a high-performance liquid chromatography (HPLC) system, MAGIC 2002 (AMR). The flow rate of the mobile phase was 1 μL/min using a MAGIC Variable Splitter. The solvent composition of the mobile phase was programmed to change in 50 min cycles with varying mixing ratios of solvent A [2% (v/v) CH_3CN and 0.1% (v/v) HCOOH] to solvent B [90% (v/v) CH_3CN and 0.1% (v/v) HCOOH]. Next, the peptides were eluted with a linear gradient from 0% to 50% solvent B. Purified peptides were introduced from HPLC to a hybrid quadrupole time-of-flight mass spectrometer, Q-star (Applied Biosystems, CA, USA) via an attached FortisTip (AMR). Mascot search engine (Matrix Science, London, UK) was utilized to identify proteins from the mass and tandem mass spectra of the peptides.

Western blotting

Western blotting was performed as described elsewhere [13]. Briefly, cells were lysed with a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, and protease inhibitors. The cell lysates were centrifuged at 15,000g for 20 min at 4 °C, and the supernatant was treated with SDS sample buffer following separation using electrophoresis on a 10% (w/v) gel. Proteins were transferred to a polyvinylidene difluoride membrane and the membrane was incubated with anti-SUMO-2/3 antibody (ZYMED, San Francisco, USA) diluted at 1:1000,

[1] *Abbreviations used:* NBCCS , nevoid basal carcinoma syndrome; 2D, two-dimensional; PCNA, proliferating cell nuclear antigen; 2D-PAGE, two-dimensional differential gel electrophoresis.