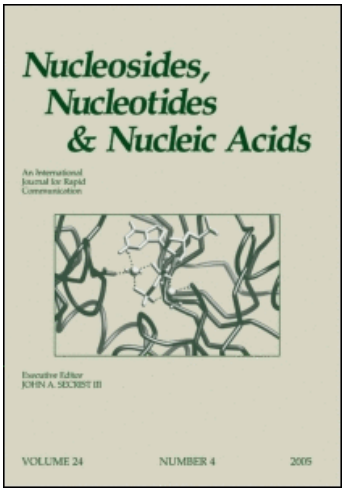

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ASSOCIATION OF NUCLEAR MEMBRANE PROTEIN LAMIN B1 WITH NECROSIS AND APOPTOSIS IN CELL DEATH INDUCED BY 5-FLUORO-2′-DEOXYURIDINE

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□ *We report that anticancer 5-fluoro-2′-deoxyuridine (FUdR) shows cytotoxicity against mouse cancer cell line FM3A, using a progeny clone F28-7 and its variant F28-7-A. In this process, the cell-death morphology is different between F28-7 and F28-7-A cells, that is, necrosis in F28-7 but apoptosis in F28-7-A cells. In the proteomic analysis of these cells before their exposure to FUdR, the nuclear inner-membrane protein lamin B1 is up-regulated in F28-7 but not in F28-7-A, suggesting that lamin B1 may possess a function to regulate the morphology of cell-death. A knockdown of lamin B1 expression in F28-7 cells was performed by use of the small interfering RNA technique, resulting in a decrease of the lamin B1-expression level down to the level in F28-7-A. Remarkably, the FUdR-induced death morphology of this knocked-down F28-7 was apoptosis, definitely different from the necrosis that occurs in the FUdR-treated original F28-7. Thus, the swelling feature for the necrosis was no longer observable, and instead cell shrinkage typical of apoptosis took place in almost all the cells examined. This finding suggests a new role for lamin B1 as a regulator in cell death.*

Keywords 5-Fluoro-2′-deoxyuridine; necrosis; apoptosis; lamin B1

INTRODUCTION

Two general pathways for cell death have been defined, necrosis and apoptosis. Depending on cell type, cellular context, or stimulus, a cell is destined to necrosis or to apoptosis.[1] Necrosis is characterized by swelling of the cell accompanying enlargement of the organelles in it, followed by disruption of the cell membrane, resulting in cell lysis.[1,2] In contrast,

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apoptosis is morphologically characterized by membrane blebbing, shrinking of the cell and its organelles, and internucleosomal DNA degradation, followed by disintegration of the cell.[3,4] However, previous studies of Leist and Nicotera[5] and Kakutani et al.[6] suggest that some early events in the death program may be common in the two types of cell death, and that downstream events may contribute to the guiding of cells toward necrosis or apoptosis. It is important to elucidate how a cell is guided toward either necrosis or apoptosis.

Recent studies indicate that signalling pathways, such as death receptors, kinase cascades, and mitochondria participate in both of these cell-death processes.[7] We have now explored the possibility that by modulating these pathways, a switch between necrosis and apoptosis may occur.

5-Fluoro-2′-deoxyuridine (FUdR), a potent anticancer agent, exerts its effect by inhibiting thymidylate synthase, an essential machinery for DNA synthesis in cell proliferation.[8,9] In our previous study on the action of FUdR against mouse mammary tumor FM3A cell line, using the original clone F28-7[6] and its variant F28-7-A cells,[6] we noticed that the FUdR-treatment can induce in F28-7 cells a breakdown of DNA into chromosome-sized fragments leading to necrosis, but, on the other hand, in F28-7-A a more extensive DNA cleavage into oligonucleosome-sized fragments and subsequent development of apoptosis are observed.[6] However, the process of regulation in the FUdR-induced necrosis and apoptosis are still unclear. Recently, we investigated the pattern of differentially expressed proteins in these cells by the proteomic analysis using two-dimensional gel electrophoresis and mass spectrometry. With this analysis, it was shown that at the untreated stage the nuclear membrane-constituent protein lamin B1 is 2.4-fold higher in F28-7, compared with F28-7-A cells (results to be published elsewhere). Lamin B1 is one of the nuclear lamins and a key structural component of the nuclear lamina, an intermediate filament meshwork that lies beneath the inner nuclear membrane. It is known that the nuclear lamins play a crucial role in fundamental cellular processes, including nuclear organization, chromatin segregation, DNA replication, and gene expression.[10–14]

Here, we report that lamin B1 may regulate necrotic and apoptotic morphology induced by FUdR. Thus, when a knockdown of lamin B1 expression in F28-7 cells by small interfering RNA (siRNA) was performed, the upregulation of lamin B1 disappeared in these cells. We then explored whether this lack of upregulation may affect the FUdR-induced cell death morphology. Remarkably, the cell death morphology was no longer the necrosis but it took a distinctly apoptotic feature. These findings suggest an interesting possibility that lamin B1 regulates the cell death pattern.

MATERIALS AND METHODS

Cells, Cell Culture, and Reagents

The two cell clones derived from a mouse mammary tumor FM3A cell line, the wild-type F28-7 and its variant F28-7-A cells, used in this study have been described previously.[6] The cells were cultured in ES medium (Nissui Pharmaceuticals, Japan) supplemented with 2% fetal bovine serum (Gibco, USA) and 0.03% L-glutamine (Wako, Japan) in a humidified atmosphere with 5% CO_2 at 37°C. Under these conditions, the doubling time was approximately 12 hours. Cell viability was estimated with a hemocytometer by means of trypan blue dye-exclusion.

5-Fluoro-2′-deoxyuridine was obtained from Sigma (USA). The lamin B1-siRNA was Mm_Lmnb1_1_HP siRNA, Catalog number: SI01090831, Sense; r(CGU UGU AAG AUG UGA AUU A)dTdT, Antisense; r(UAA UUC ACA UCU UAC AAC G)dTdG, and the nonsilencing siRNA was AllStars Negative Control siRNA, Catalog number: 1027280, Sense; unpublished, Antisense; unpublished. Both were purchased from Qiagen (Germany). The primary antibodies; mouse monoclonal anti-lamin-B1 and rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Zymed Laboratories (USA) and Trevigen (USA), respectively. The secondary antibodies; anti-mouse IgG horseradish peroxidase-linked whole antibody and anti-rabbit IgG horseradish peroxidase-linked whole antibody were from GE Healthcare (UK). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Invitrogen (USA).

Transfection

Exponentially growing 2×10^5 F28-7 cells were suspended in 75 μl siPORT electroporation buffer (Ambion, USA) containing lamin B1 or nonsilencing siRNA (final concentration 1×10^{-7} M) and introduced into 0.1 cm gap electroporation cuvette (Bio-Rad, USA). Cells were then electroporated using the Bio-Rad Gene Pulser Xcell at voltage 0.15 kV, pulse length 1,000 μs, and number of pulse 1. After electroporation, cells were plated at 5×10^4 cells/ml in fresh ES medium in tissue culture flasks. Forty-eight hours after the electroporation, cells were used for further experiments.

Western Blot Analysis

Cells were washed in phosphate-buffered saline (PBS) and then whole cell lysates were prepared using Laemmili sample buffer (Bio-Rad). Proteins (5×10^4 cells equivalent per lane, respectively) were subsequently fractionated under reducing conditions by 7.5% SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane

(Millipore, USA). The blotted membrane was blocked for 1 hour with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 and then immunoblotted overnight at 4°C using the respective primary antibody. The membrane was then incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibody, and the protein bands were visualized using an ECL plus western blotting detection system (GE Healthcare). Protein expression was quantified using VersaDoc imaging system (Bio-Rad). The following antibodies were used: anti-lamin B1 antibody (1:1,500), anti-GAPDH antibody (1:10,000), anti-mouse IgG horseradish peroxidase-linked whole antibody (1:20,000), anti-rabbit IgG horseradish peroxidase-linked whole antibody (1:20,000).

Morphological Changes

Cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS at 4°C for 30 minutes. Then, the fixed cells were washed with PBS and stained with DAPI in PBS. The stained cells were spread on a slide and mounted with coverslips. Cell morphology was observed by Olympus BX61 fluorescence microscopy.

RESULTS AND DISCUSSION

In our proteomic analysis, lamin B1 was identified as an up-regulated protein in F28-7, in contrast to the normal level in the variant cell strain F28-7-A (results to be published elsewhere). Recent studies from several laboratories indicate that lamin B1 and other lamins play a role in nuclear architecture, DNA replication, and gene expressions.[10–14] The possibility that lamin B1 may be associated with the differential patterns of cell death morphology observable in the treatment with FUdR is now investigated. The Western blot analysis of the cells before the treatment with FUdR revealed that lamin B1 protein level is 1.8-fold higher in F28-7, in comparison to that in F28-7-A cells (Figure 1A). Thus, the greater enhancement in the F28-7 over the F28-7-A observed in the proteomic analysis was confirmed by this Western blot analysis. To test if a downregulation of endogenously expressed lamin B1 in F28-7 cells can modulate FUdR-induced necrosis, we carried out a knockdown of the expression in F28-7 cells by using lamin B1 siRNA. Transfection efficiencies at higher than 80% were obtained by the electroporation (data not shown). Western blot analysis performed for cell extracts at 48 hours after the transfection indicates that the siRNA treatment resulted in a reduction of the lamin B1 protein levels, while the GAPDH protein levels as a control showed no change (Figure 1B). The level of lamin B1 protein expression in F28-7 became the same as that in F28-7-A cells. Another control experiment in which a nonsilencing siRNA

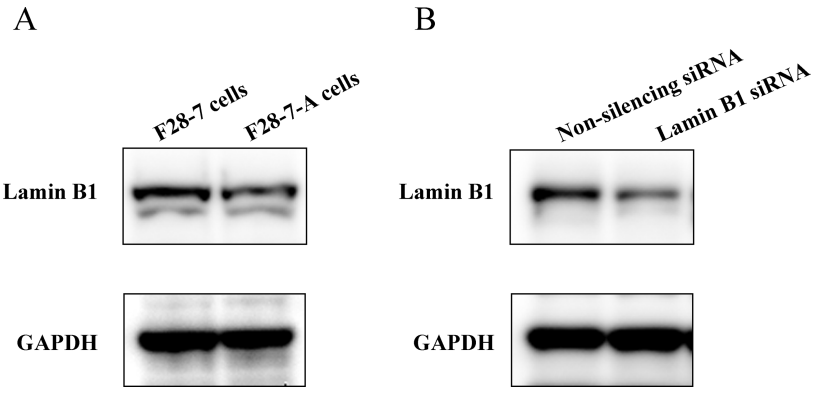

FIGURE 1 Knockdown of lamin B1 by RNA interference. A) Whole cell lysates were prepared from F28-7 and F28-7-A cells. Expression of lamin B1 and GAPDH proteins were examined by Western blot analysis. Expression of GAPDH was used as an internal control. The patterns shown are results obtained in one set of experiments. Three independent sets of experiments were performed gaving similar results. B) F28-7 cells were transfected with nonsilencing siRNA, and lamin B1 siRNA. Forty-eight hours after the transfection, the levels of expression of lamin B1 and those of GAPDH were examined by Western blot analysis.

was administered showed no effect on the expression of lamin B1 or GAPDH (Figure 1B). The cell viability at 48 hours after the transfection was 98% with the vehicle, 98% with the nonsilencing siRNA, and 96% in the lamin B1 siRNA-trensfected cells. In addition, nonsilencing siRNA and lamin B1 siRNA themselves had no impact on the cell morphology, if no FUdR administration is performed (Figure 2, upper diagram). The knockdown of lamin B1 in the F28-7 cells did not change the cell viability, if FUdR was not given.

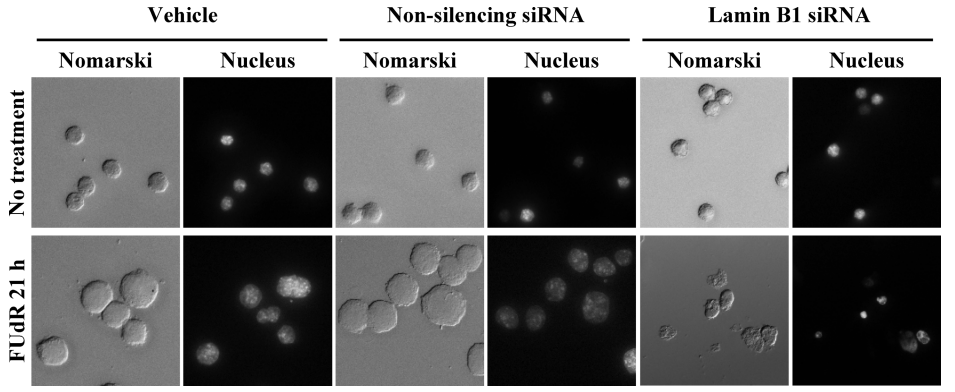

FIGURE 2 Lamin B1 knockdown shifts FUdR-induced necrotic morphology to apoptotic morphology. Forty-eight hours after transfection with the vehicle, the nonsilencing siRNA, or the lamin B1 siRNA, the F28-7 cells were treated with or without 1×10^{-6} M FUdR for 21 hours and then stained with DAPI as described under Materials and Methods. Morphological changes were analyzed under a fluorescence microscope at 400 × magnification. The results are typical of the results of three experiments that gave similar results.

Next, we explored the morphology in the lamin B1-knockdown F28-7 cells on treatment with 1×10^{-6} M FUdR. At 21 hours after the treatment was initiated, the controls given vehicle or nonsilencing siRNA showed the cytoplasmic swelling, a hallmark for necrosis. The lamin B1 siRNA transfected cells, in contrast, showed a typical apoptotic morphology; the membrane blebbing and the formation of apoptotic bodies (Figure 2, at the bottom). At this period of treatment, the cell viability was 26% with the vehicle, 24% with the nonsilencing siRNA, and 23% with the lamin B1 siRNA transfection. Furthermore, almost all the dyeing cells underwent apoptosis after the lamin B1 siRNA transfection with subsequent FUdR treatment. These observations suggest that lamin B1 participates in modulating the process of the FUdR-induced necrosis and apoptosis. It would be important to further investigate the mechanisms involved in the lamin B1-control of the necrosis and apoptosis in the process of FUdR treatment. Our present work may contribute to the understanding of the mechanisms regulating necrosis and apoptosis.

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