DNA damage induces p21 protein expression by inhibiting ubiquitination in ML-1 cells

Kunihiko Fukuchi a,*, Shigeru Tomoyasu b, Tsuyoshi Nakamaki b, Nobuyoshi Tsuruoka b, Kunihide Gomi a

a Department of Clinical Pathology, Showa University, School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan
b Department of Hematology, Showa University, School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

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Abstract

We previously reported that deferoxamine, an iron chelating agent, induced p53 and cell accumulation in the G1 phase of ML-1 cells in the same way as the DNA damaging agent, etoposide. Etoposide treatment increased expression of the p21 gene, a cyclin kinase inhibitor, at both the mRNA and protein levels. However, deferoxamine treatment only increased the p21 mRNA level without the appearance of a detectable protein product. A substrate for cyclin kinase, pRB, was unphosphorylated by etoposide treatment, but remained unaffected by deferoxamine, indicating that p21 was functional after etoposide, but not after deferoxamine treatment. Therefore, in the present study, we investigated the involvement of the ubiquitin proteasome pathway in post-transcriptional regulation of p21. By the addition of lactacystin, a proteasome inhibitor, to deferoxamine treatment, the level of unubiquitinated p21 protein product was similar to that induced by etoposide treatment, and the ubiquitinated p21 bands became apparent. After etoposide treatment, the level of ubiquitinated p21 was diminished and a high level of unubiquitinated p21 expression was observed. We concluded that (1) efficient expression of p21 protein requires inhibition of the ubiquitin-proteasome pathway, and (2) DNA Damage inhibits the ubiquitination of p21.

Keywords: p21; Ubiquitin; Proteasome; DNA Damage; Deferoxamine

1. Introduction

Cell cycle transition is regulated by the coordinated expression of many components, such as p53, cyclin, CDKs, and CDK inhibitors [1,2]. Expression of the relevant genes is controlled by transcription, mRNA stability, or protein stability.

A CDK inhibitor, p21, inhibits the cell cycle through its interaction with cyclin-CDK complexes [3,4]. Expression of p21 appears to be regulated by both transcriptional and post-transcriptional mechanisms. At the transcriptional level, p21 mRNA is induced by wild-type p53 [5], and also induced independently of p53 in several situations, including normal tissue development [6], cellular differentiation [7–9], the presence of DNA damaging agents [10–12], TGF-β treatment [13,14], retinoic acid treatment [15], vitamin D3 treatment [16] and interferon treatment [17]. Post-transcriptional regulation of p21 expression has been demonstrated: stabilization of p21 mRNA during hematopoietic differentiation [18] or
after TNFα treatment [19], or okadaic acid-induced p21 expression without mRNA stabilization [20].

The involvement of ubiquitin-proteasome degradation of several cell cycle-regulatory proteins, such as cyclin [21], p27 [22], or p53 [23], has been reported. Levels of wild-type p53 are quite low due to a short protein half-life. However, p53 protein accumulates after the treatment of cells with various DNA damaging agents via different mechanisms, such as increased translation [24] and protein stabilization [25]. Maki and Howley demonstrated that UV irradiation of cells led to the stabilization of wild-type p53 through the loss of p53 ubiquitination [26]. The p53 function that induces apoptosis was accomplished with the stabilization and accumulation of p53 protein after treatment with proteasome inhibitor [27]. Recently, the regulation of p21 expression by the ubiquitin-proteasome degradation pathway was postulated after demonstrating in vivo ubiquitination [25,26,28].

We previously reported a cellular response to treatment with deferoxamine B mesylate (DFO), an iron-chelating agent, using a myelocytic leukemia cell line, ML-1, which contains wild-type p53 [30,31]. It is generally thought that DFO inhibits the iron-requiring enzyme ribonucleotide reductase, thereby altering the supply of deoxyribonucleotides and decreasing DNA synthesis [29]. We observed the increase of p53 protein followed its accumulation in the G1 phase after treatment of ML-1 cells with DFO [30,31]. The accumulation of p53 protein by DFO treatment was similar to that observed after treatment with etoposide, a topoisomerase II inhibitor [32]. Etoposide and lactacystin were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 1 × 10^{-3} M and 1 × 10^{-2} M, respectively. DFO, hydroxyurea and AraC were dissolved in H_{2}O at a concentration of 1 × 10^{-1} M. Exponentially growing ML-1 cells were seeded at a concentration of 2 × 10^{5}/ml; 24 h later, various agents were added to the medium, which was then incubated for an additional 6 h or 24 h, or irradiated with 15 or 30 Gy of γ-rays, then incubated for an additional 6 h.

2. Materials and methods

2.1. Cell culture and treatment

A myelocytic leukemia cell line, ML-1 cells, supplied by the Health Science Research Resource Bank (HSRRB), was cultured in RPMI 1640 containing 10% fetal calf serum. Deferoxamine B mesylate (DFO, M_r 656.8) was obtained from Ciba-Geigy (Basel, Switzerland). Etoposide, hydroxyurea, and AraC were purchased from Sigma (USA). Lactacystin was kindly provided by Dr. S. Omura of the Kitasato Institute [34]. Etoposide and lactacystin were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 1 × 10^{-1} M and 1 × 10^{-2} M, respectively. DFO, hydroxyurea and AraC were dissolved in H_{2}O at a concentration of 1 × 10^{-1} M. Exponentially growing ML-1 cells were seeded at a concentration of 2 × 10^{5}/ml; 24 h later, various agents were added to the medium, which was then incubated for an additional 6 h or 24 h, or irradiated with 15 or 30 Gy of γ-rays, then incubated for an additional 6 h.

2.2. RNA extraction and Northern blot analysis

Total cellular RNA extraction and Northern blot hybridization were performed as described previously [31]. To extract polysomal RNA, cells were lysed in 0.5% Nonidet P-40, 1 × 10^{-2} M Tris-HCl pH 7.4, 1.5 × 10^{-3} M NaCl, 1.5 × 10^{-3} M MgCl_{2}, and 2 × 10^{-2} M ribonucleoside vanadyl complexes (R3380 Sigma) on ice for 30 min, then the nuclei were precipitated with 3000 rpm (600×g) for 10 min at 4°C. The supernatant was centrifuged at 12 000 rpm (8000×g) for 10 min at 4°C to precipitate the mitochondrial fraction and the resulting supernatant was centrifuged at 60 000 rpm (105000×g) at 4°C for 60 min to pellet the microsome fraction. RNA from the microsome fraction was used as polysomal RNA. RNAs were extracted from each cellular fraction by the guanidine method [35]. p21 cDNA (provided by Dr. A. Noda, Meiji Cell Technology Center, Japan), and β-actin DNA were digoxigenin-labeled by the random primer method [35]. The hy-
bridized probes were immunodetected with anti-digoxigenin antibody, then visualized with the colorimetric substrates, 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/X-phosphate). The level of expression was normalized by comparison with β-actin mRNA levels measured by a densitometer.

2.3. Protein extraction and immunoblotting

Cells (1 × 10^7) were lysed in 1 ml of lyse buffer (1% Nonidet P-40, 1 × 10^{-2} M sodium phosphate pH 7.2, 1.5 × 10^{-1} M NaCl, 1 × 10^{-3} M EDTA, 5 × 10^{-2} M sodium fluoride, 1 × 10^{-2} M sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 5 μg/ml phenylmethylsulfonyl fluoride). After incubation on ice for 30 min, the lysate was clarified by ultracentrifugation at 30,000 rpm (30,000 × g) for 30 min at 4°C. The cell lysate was immunoprecipitated with anti-p21 polyclonal antibody (15431E PharMingen, CA, USA) or anti-p21 monoclonal antibody (2G12, PharMingen) with 0.01% SDS and 0.1% sodium deoxycholate, after which the precipitate was separated by SDS-polyacrylamide gel electrophoresis and blotted on a PVDF membrane (BioRad, CA, USA), then incubated with anti-p21 monoclonal antibody (2G12, PharMingen) or anti-ubiquitin polyclonal antibody (N19 Santa Cruz, CA, USA). The blot was washed to remove unbound antibody and incubated with a biotinylated anti-mouse IgG for p21 detection by monoclonal antibody or biotinylated anti-rabbit IgG for the detection of ubiquitinated p21 by polyclonal antibody. Following this incubation, the membrane was again washed and incubated with the streptavidine-biotinylated alkaline phosphatase complex, which binds to the biotin of the second antibody. After washing thoroughly, NBT/X-phosphate substrate was used to visualize the bands.

3. Results

3.1. p21 mRNA and protein product

In ML-1 cells, p21 mRNA expression increased after 6 h treatment with 1 × 10^{-5} M etoposide, 1 × 10^{-5} M AraC, 1 × 10^{-4} M DFO, and 1 × 10^{-3} M hydroxyurea and also increased 6 h after treatment with 15 Gy or 30 Gy γ-irradiation (Fig. 1). The level of expression was normalized by comparison with β-actin and indicated as relative intensity. UT, untreated; 15 Gy, 15 Gy γ-irradiation; 30 Gy, 30 Gy γ-irradiation; Etop, etoposide; HU, hydroxyurea; DFO, deferoxamine.

Fig. 1. p21 mRNA expression after treatment with various agents. Total RNA was extracted from cells 6 h after 15 Gy and 30 Gy γ-irradiation and the addition of 1 × 10^{-5} M etoposide, 1 × 10^{-4} M hydroxyurea, 1 × 10^{-4} M DFO and 1 × 10^{-3} M AraC. Twenty micrograms of total RNA were subjected to electrophoresis on formaldehyde agarose gel, transferred to a Hybond N+ membrane, and hybridized with digoxigenin-labeled probes. The level of expression was normalized by comparison with β-actin and indicated as relative intensity. UT, untreated; 15 Gy, 15 Gy γ-irradiation; 30 Gy, 30 Gy γ-irradiation; Etop, etoposide; HU, hydroxyurea; DFO, deferoxamine.

3.2. p21 mRNA in polysomal RNA

In order to examine whether or not p21 mRNAs were properly translocated to the polysomes for translation, polysomal RNA and nuclear RNA were fractionated and analyzed by Northern blotting. In polysomes, an enhanced level of p21 mRNA was detected after treatment with etoposide or DFO, similar to that observed with total RNA (Fig. 3). The p21 mRNAs were not detected in the
supernatant after centrifugation at 60,000 rpm (105,000 x g) (data not shown). These findings strongly suggest post-transcriptional regulation of p21 expression.

3.3. Proteasome inhibitor treatment

To analyze the association of degradation by proteasome with the regulation of p21 expression, we examined the effect of the proteasome inhibitor, lactacystin, at a concentration of 2 x 10^{-6} M. First, the p21 mRNA level was examined after treatment with 2 x 10^{-6} M lactacystin for 6 h and 24 h (Fig. 4A). The level of p21 mRNA was not affected after treatment with DMSO used to dissolve the lactacystin (Fig. 4A).

Fig. 3. p21 mRNA in the polysome fraction. ML-1 cells were treated with 1 x 10^{-5} M etoposide and 1 x 10^{-4} M DFO for 6 h. Total cellular RNA, nuclear RNA and polysomal RNA were extracted separately and analyzed by Northern blot hybridization. UT, untreated; DFO, deferoxamine; Etop, etoposide.

3.3. Proteasome inhibitor treatment

To analyze the association of degradation by proteasome with the regulation of p21 expression, we examined the effect of the proteasome inhibitor, lactacystin, at a concentration of 2 x 10^{-6} M. First, the p21 mRNA level was examined after treatment with lactacystin. The level of p21 mRNA was slightly increased after treatment with 2 x 10^{-6} M lactacystin for 6 h and 24 h (Fig. 4A). The level of p21 mRNA was not affected after treatment with DMSO used to dissolve the lactacystin (Fig. 4A).
Next, we examined the expression of p21 protein products by immunoblot analysis after immunoprecipitation of the cell lysate with anti-p21 monoclonal antibody. Treatment with DFO for 6 h induced p21 mRNA, but not the p21 protein product (Fig. 2). After incubation with lactacystin and DFO together for 6 h, the p21 band did not appear (data not shown). We therefore examined the p21 protein at the other time point. After 24 h incubation with DFO and lactacystin, the p21 band clearly appeared (Fig. 4B). When ML-1 cells were incubated with lactacystin alone, a weak p21 band was observed (Fig. 4B). This band may have been due to stabilization of the p21 protein product and slight upregulation of p21 mRNA expression at 24 h incubation.

Immunoblot analysis detected p21 protein products after treatment of ML-1 cells with lactacystin and AraC or lactacystin and hydroxyurea (data not shown).

We surmise that the p21 mRNA induced by DFO treatment in ML-1 cells was utilized for translation.

3.4. Decrease in ubiquitination of p21 after treatment with DNA damaging agents

Since the effect of lactacystin became apparent after 24 h treatment, we performed Western blot analysis for the ubiquitinated p21 protein after 24 h incubation. The anti-ubiquitin polyclonal antibody detected ubiquinated p21 on the p21 immunoprecipitate as bands of approx. 30, 39 and 47 kDa respectively in ML-1 cell lysate after treatment with DFO and lactacystin (Fig. 5). These bands corresponded to species of p21 conjugated with one, two or three ubiquitin molecules ($M_r$ 8600), respectively. These bands were found to have the same size as those detected by anti-p21 monoclonal antibody in cells treated with DFO and lactacystin (Fig. 5, right panel). By treatment with lactacystin alone, ubiquitinated p21 bands were observed to a lesser extent. The ubiquitinated p21 band was not detected in untreated, DFO, or etoposide-treated (6 h and 24 h) cells.

4. Discussion

In this study, we analyzed the involvement of the ubiquitin-proteasome degradation pathway in post-transcriptional regulation of p21 expression.

The p21 mRNA induced in ML-1 cells after treatment with DNA damaging agents, etoposide or $\gamma$-irradiation, or non-DNA damaging agents, DFO or hydroxyurea, were located in the polysome fraction. However, the p21 protein product was observed only after treatment with DNA damaging agents. The discrepancy between the mRNA level and protein product level suggests that p21 expression is post-transcriptionally regulated.

When ML-1 cells were treated with DFO and lactacystin together, a high level of p21 protein product was detected. The data showed that p21 mRNAs induced by DFO treatment in ML-1 cells were translated efficiently and the protein product remained intact with the addition of proteasome inhibitor. These results clearly suggested that the p21 product is degraded by the ubiquitin-proteasome system, and that the regulation of translational initiation of p21 mRNA is not likely to be a major regulatory mechanism in this case. Accordingly, these findings sug-
gested that p21 expression was regulated at the post-transcriptional level.

Further detection of ubiquitinated p21 in ML-1 cells after treatment with proteasome inhibitors supports the notion that the ubiquitin-proteasome pathway is involved in the regulation of p21 expression. The p21 band observed on immunoblotting after treatment with proteasome inhibitors had migrated as unubiquitinated p21. There is some evidence that the proteasome inhibitor, MG132, or lactacystin treatment of cells increased the level of unubiquitinated p53 [36,37]. Hence, we surmise that proteasome inhibitor treatment, especially with lactacystin, inhibits proteasome activity, and somehow inhibits the ubiquitination of p21.

Ubiquitinated p21 bands were observed in cells treated with proteasome inhibitors. Treatment with the DNA damaging agent, etoposide, increased the p21 mRNA and protein product, but the level of ubiquitinated p21 diminished in ML-1 cells. This suggested the existence of mechanisms for inhibiting ubiquitin ligase activity or activation of deubiquitination by DNA damaging agents.

DNA damage regulates the cell cycle check point through various mechanisms. DNA damage from ionizing radiation activates the ATM gene product to trigger the c-Abl tyrosine kinase activity independently from p53 status [38,39]. Ionizing radiation induces the functional interaction between c-Abl kinase and DNA-dependent protein kinase to associate DNA double strand breaks [40]. Signal transduction pathway is considered to convert DNA damage into intracellular signals. Phosphorylation of target protein is reported to be a signal for ubiquitination in the case of p27 [41], c-Jun [42], I-κB [43], and STAT-1 [44]. In the future, we will examine the phosphorylation of p21 after treatment with a DNA damaging agent.

We concluded that (1) efficient expression of p21 protein requires inhibition of the ubiquitin-proteasome pathway, and (2) DNA damage inhibits the ubiquitination of p21.

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