Involvement of Cdk Inhibitor p21(WP1/CIP1) in G2/M Arrest of Human Myeloid Leukemia U937 Cells by \(N\)-Methyl-\(N\)\(^{-}\)Nitro-\(N\)\(^{-}\)Nitrosoguanidine

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In this paper, to elucidate the further mechanisms of \(N\)-methyl-\(N\)\(^{-}\)nitro-\(N\)\(^{-}\)Nitrosoguanidine (MNNG)-induced growth arrest, we investigated the effect of MNNG on cell cycle and proliferation in U937 cells, a p53-null human myeloid leukemia cell line. It was found that MNNG causes an arrest at the G2/M phase of the cell cycle and induces apoptosis, which is closely correlated to inhibition of cyclin B1 and cyclin-dependent kinase (Cdk) 2-associated kinase activities. MNNG treatment increased protein and mRNA levels of the Cdk inhibitor p21(WAF1/CIP1), and activated the reporter construct of a p21 promoter. By using p21 promoter deletion constructs, the MNNG-responsive element was mapped to a region between 113 and 61 relative to the transcription start site. These data indicate that in U937 cells MNNG can circumvent the loss of wild-type p53 function and induce critical downstream regulatory events leading to transcriptional activation of p21. Present results indicate that the p53-independent up-regulation of p21 by MNNG is likely responsible for the inhibition of cyclin/Cdk complex kinase activity rather than the down-regulation of cyclins and Cdk expression. These novel phenomena have not been previously described and provide important new insights into the possible biological effects of MNNG.

Key words : \(N\)-methyl-\(N\)\(^{-}\)Nitro-\(N\)\(^{-}\)Nitrosoguanidine (MNNG), U937, G2/M arrest, p21

Introduction

Genotoxic events such as ultra violet and alkylating agents can activate various signaling pathways that serve, for example, to activate DNA repair mechanisms, halt cell cycle progression and/or trigger advancement into apoptosis, a programmed cell death. Although all genotoxins produce such general responses, the mechanisms that govern response to divergent forms of DNA damage are potentially diverse themselves. Alkylating agents are an important class of anticancer drugs used in the chemotherapy [3,20,29]. They produce a variety of lesions by covalently transferring the alkyl group to oxygen and nitrogen atoms in DNA, and the damage is repaired by the DNA mismatch repair and/or base-excision repair pathway [6,35].

Among them, the monofunctional alkylating agent \(N\)-methyl-\(N\)\(^{-}\)Nitro-\(N\)\(^{-}\)Nitrosoguanidine (MNNG) is known to cause growth arrest at the G2/M checkpoint and apoptosis in various human cancer cell lines [1,7,15,16,21,31,32]. Previous reports showed that the treatment of human cancer cells with MNNG results in p53 phosphorylation on serine residues, and these phosphorylation events depend on the presence of functional protein complexes involved in mismatch repair [2,13,17]. These results implicate action of the mismatch repair system in the initial step of a damage-signaling cascade that can lead to cell-cycle checkpoint activation or apoptosis in response to DNA damage [2,17,22]. To date, despite accumulated data, the molecular mechanism of MNNG-induced antiproliferative action on the cell cycle of human cancer cells is poorly understood.

The progression of eukaryotic cells through the cell cycle is orchestrated by sequential activation and inactivation of the cyclin-dependent kinases (Cdks), which is associated with their respective cyclin subunits. G1 progression and G1/S transition are regulated by Cdk4/Cdk6 which assembles with D-type cyclins in mid-G1 and Cdk2 which combines later with cyclin E. While Cdk2 controls the S-phase when associated with cyclin A, G2/M transition is regulated by Cdc2 in combination with cyclins A and B [11,33,36]. In addition, the relative balance between the cellular concentrations of low molecular weight Cdk inhibitors also regulates the cell cycle progression. Two gene families of mammalian Cdk inhibitors have been identified to date: one group is the Cdk-interaction protein/Cdk inhibitor (CIP/kip) family, including p21, p27 and p57, and has a broad
specificity; and the other group acts as inhibitors of the Cdk4 (INK4) family including p15, p16, p18 and p19. INK4 binds only to Cdk4 and Cdk6, while CIP/KIP targets a broader range of substrates, including Cdk1, Cdk2, A- and B-dependent kinases [11,25,34].

In this study, we have shown that MNNG inhibited the cell growth and induced apoptotic cell death in human myeloid leukemia U937 cells, which are deleted and mutated by the p53 gene [9]. Subsequent experiments indicated that U937 cells were arrested at the G2/M checkpoint by MNNG, which was accompanied by the inhibition of cyclin B1 and Cdk2-associated kinase activity and transcriptional up-regulation of p21 in a p53-independent manner.

Materials and Methods

Cell culture and viability assay

Human myeloid leukemia U937 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL) at 37°C and 5% CO2. MNNG was purchased from Sigma Chemical Co. (St. Louis, MO), dissolved in distilled water. Cells were cultured in the absence and presence of variable concentrations of MNNG for the indicated times. Measurement of cell viability was determined using a MIT assay, which is based on the conversion of MIT to MIT-tetrazolium by mitochondrial enzyme.

Flow cytometry analysis

Cells were harvested and washed once with cold phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol and then stored at 4°C. Prior to analysis, the cells were washed once again with PBS, suspended in 1 ml of a cold propidium iodide (PI) solution containing 100 μg/ml RNase A, 50 μg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and then incubated on ice for an additional 30 min in the dark. Next, flow cytometric analyses were conducted using a flow cytometer (FACS Caliber, Becton Dickinson, San Jose, CA) and the CellQuest software was then used to determine the relative DNA content based on the presence of a red fluorescence.

Annexin-V and PI staining

Cells were collected, washed with cold PBS and then suspended in annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2 according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA). Aliquots containing cells were incubated with annexin-V FITC, mixed, and incubated for 15 min at room temperature in the dark. PI at a final concentration of 5 μg/ml was added to identify necrotic cells. Apoptotic cells were measured using a fluorescence-activated cell sorter analysis in a flow cytometer.

RNA extraction and reverse transcription-PCR

Total RNA was prepared using an RNaseasy kit (Qiagen, La Jolla, CA) and primed with random hexamers to synthesize the complementary DNA using AMV reverse transcriptase (Amersham Corp, Arlington Heights, IL) according to the manufacturer’s instructions. A polymerase chain reaction (PCR) was carried out using a Mastercycler (Eppendorf, Hamburg, Germany) with the primers shown in Table 1. The following conditions were used for the PCR reactions: 1× (94°C for 3 min); 35× (94°C for 45 sec; 58°C for 45 sec; and 72°C for 1 min) and 1× (72°C for 10 min). The amplification products obtained by PCR were separated electrophoretically on 1% agarose gel and visualized by ethidium bromide (EtBr) staining.

Gel electrophoresis and Western blot analysis

The cells were harvested, lysed, and the protein concentrations were quantified using a Bio Rad protein assay (BioRad Lab., Hercules, CA), according to the procedure reported by the manufacturer. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gel and transferred to a nitrocellulose

Table 1. Gene-specific primers for RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of primers</th>
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<tbody>
<tr>
<td>Cyclin A sense</td>
<td>5'-TCC-AAG-AGG-ACC-AGG-AGA-ATA-TCA-3'</td>
</tr>
<tr>
<td>antiseence</td>
<td>5'-TCC-TCA-TGG-TAG-TCC-GGT-ACI-TCA-3'</td>
</tr>
<tr>
<td>Cyclin B1 sense</td>
<td>5'-AAG-ACT-TTT-AAA-CIT-TGG-CTT-GG3'</td>
</tr>
<tr>
<td>antiseence</td>
<td>5'-CTT-TGT-AAG-CCC-TTG-ATT-TAC-CAI-G-3'</td>
</tr>
<tr>
<td>Cdk2 sense</td>
<td>5'-CGG-GAT-TCA-GAA-ATI-GAT-CA-3'</td>
</tr>
<tr>
<td>antiseence</td>
<td>5'-TGT-CAG-AAA-GCT-ACA-TCI-TG-3'</td>
</tr>
<tr>
<td>Cdk2 sense</td>
<td>5'-CTT-CTC-TCC-CAT-CTI-AG-3'</td>
</tr>
<tr>
<td>antiseence</td>
<td>5'-CTC-CCT-CCA-GTA-GCA-AAG-AT-3'</td>
</tr>
<tr>
<td>p21 sense</td>
<td>5'-GCT-GGG-ACT-GAG-ACT-GCT-3'</td>
</tr>
<tr>
<td>antiseence</td>
<td>5'-AGC-CCT-CTI-CTG-GTA-GCT-3'</td>
</tr>
<tr>
<td>p27 sense</td>
<td>5'-AGG-CTA-GTG-CGG-GAT-ATG-GA-3'</td>
</tr>
<tr>
<td>antiseence</td>
<td>5'-AGA-CCC-GGC-TGG-TAC-GAC-3'</td>
</tr>
<tr>
<td>GAPDH sense</td>
<td>5'-GGG-AGT-CAA-CAG-ACT-GAG-3'</td>
</tr>
<tr>
<td>antiseence</td>
<td>5'-ACC-CTT-CTI-CTG-GGT-GTA-GAG-3'</td>
</tr>
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membrane (Schleicher & Schuell, Keene, NH) by electoblotting. The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibodies and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham). Primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Calbiochem (Cambridge, MA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

Immune complex kinase assay

Immune complex kinase assays were performed as described [31]. Briefly, cell lysates were incubated with primary antibody, and immune complexes were collected on protein A-Sepharose beads and resuspended in kinase assay mixture containing [γ-32P]ATP (ICN) and histone H1 (Life Technologies, Inc.) as a substrate. The proteins were separated on 10% SDS-polyacrylamide gels, and bands were detected by autoradiography.

p21 promoter-luciferase constructs and transfection assay

Four constructs of p21 promoter, the full length 2.4-kb human p21 promoter constructs as well as p21 promoter deletion constructs, p21 5ma (0 to -113), p21 5ma Δ1 (0 to -61) and p21 5ma Δ2 (full-length p21 promoter from which the region -62 to -114 was deleted) were tested [10]. For the reporter assay, cells were seeded at 2×10^5 cells per 60 mm diameter plate and transiently transfected with p21 promoter-luciferase reporter constructs using LipofectAMINE transfection reagent (Gibco BRL), as recommended by the manufacturer. Following transfeciton the cells were incubated for 12 h, the medium was exchanged, and the cells were incubated for an additional 36 h in the presence or absence of 40 ng/ml MNNG. The cells were then lysed, and luciferase activity in the lysates was assayed using a Dynatech ML1000 luminometer (Dynatech Laboratories) as previously described [32].

Results

MNNG inhibited the cell proliferation and induced G2/M arrest

To investigate the effects of MNNG on cell viability, U937 cells were treated with various concentrations of MNNG for the indicated times and subjected to MTT assays. We found that MNNG had strong inhibitory effects on cell proliferation in dose- and time dependent manner (Fig. 1A and B). To determine whether MNNG treatment resulted in the alteration of cell cycle progression, the cell cycle patterns were examined. The data demonstrated that the growth inhibiting action of MNNG was the consequence of a specific G2/M phase arrest (Fig. 1C and D). At a concentration of 40 ng/ml for 48 h, many more U937 cells (56.20%) were stationed in the G2/M phase and far fewer cells (6.54%) in the G1 phase compared with the control.

MNNG induced apoptosis

Further experiments were carried out to determine if this inhibitory effect of MNNG on cell viability is the result of apoptotic cell death. As shown in Fig. 1C and D, the addition of MNNG is seen to result in the increased appearance of cells in the sub-G1 phase, similar to the results observed for MNNG-induced loss of cell viability. Flow cytometry analysis with annexin V and PI staining was also used to determine the magnitude of apoptosis elicited by MNNG. As shown in Fig. 2, the annexin V-positive cells increased

![Fig. 1. Effect of MNNG on the proliferation of and cell cycle distribution of U937 cells. U937 cells were seeded at 3×10^5 per ml in 35 mm dish and incubated for 24 hr. The cells cultured in the absence (0, control) or in the presence of increasing concentrations of MNNG for the indicated times. (A and B) Cell viability was measured by the metabolic-dye-based MTT assay. The data shown represent the mean ± the SD of three independent experiments. (C and D) The cells grown under the same conditions were collected, fixed, and stained with PI for flow cytometry analysis. The percentages of cells in the each phase of cell cycle are presented. The data represent the average of two independent experiments.](image-url)
Fig. 2. Induction of apoptosis by MNNG in U937 cells. U937 cells were seeded at an initial density of 2.5 x 10^6 cells per 60 mm plate, incubated for 24 hr, and treated with different concentrations of WEHI for the indicated times. The cells were collected and stained with FITC-conjugated annexin-V and PI for flow cytometry analysis. The apoptotic cells were determined by counting the % of annexin V(+) /PI(+) cells and the % of annexin V(+)/PI(+) cells. The results are expressed as the mean±SD of three independent experiments.

concentration- and time dependently in the MNNG-treated U937 cells compared with the untreated control cells. These results demonstrated that the cytotoxic effects observed in response to MNNG are associated with the induction of G2/M arrest and apoptotic cell death in U937 cells, as was observed in several other cancer cell lines [1,7,15,16,21,31,32].

MNNG inhibited cyclin B1 and Cdk2-associated kinase activity

The G2/M transition is regulated by B-type cyclins which bind to and activate Cdk2 and cyclin A, which activates Cdk2. Because MNNG arrests U937 cells in G2/M, we tested whether MNNG decreased mRNA and protein levels of cyclin A, cyclin B1, Cdk2 and Cdc2, and as demonstrated in Fig. 3, there was little detectable change in the level of these mRNAs and proteins, even after 48 h of exposure to MNNG. On the other hand, Fig. 4 shows that the cyclin B1 and Cdk2-associated activities of U937 cells were markedly reduced to undetectable levels in MNNG-treated cells as assessed by enzymatic assays of specific immunoprecipitates. This data indicated that MNNG inhibited the kinase activity of cyclin/Cdk complexes by changing the activation states, rather than altering the expression of cyclins and Cdk2 proteins.

Induction of Cdk inhibitor p21 by MNNG

As Cdk activity is highly regulated by association with Cdk inhibitors, we next examined the possible up-regulation of these proteins and mRNAs in cells treated with MNNG.
Transcriptional activation of p21 promoter by MNNG

Since the p21 expression is markedly induced by MNNG, we subsequently investigated the effect of MNNG on the transcriptional regulation of p21 using p21 promoter constructs. For this study, p21 promoter-luciferase reporter constructs were transfected into U937 cells, and luciferase activity was measured in the presence and absence of MNNG. The full-length 2.4-kb promoter construct containing two p53 response elements was activated 4.96-fold by MNNG (Fig. 5C). Three deletion constructs were employed to further map this region of the p21 promoter. A construct consisting of 0 to 113 base pairs was in part responsive to MNNG. In contrast, a construct consisting of the region from 0 to 61 was non-responsive to MNNG. A deletion construct consisting of the full-length promoter minus the region 62 to 114 was also non-responsive to MNNG. These data localize the region responsive to MNNG to the sequences between 62 and 113 base pairs. Because the region between 62 and 113 contains four Sp1 binding sites [10], these data localize the MNNG-responsive region and suggest that Sp1 may play a critical role in the activation of p21 transcription in response to MNNG.

Discussion

In this study, we evaluated the ability of MNNG to inhibit the growth of human monocytic leukemia U937 cells. We found that treatment of cells with MNNG resulted in a concentration- and time dependent inhibition of cell viability, which was associated with the induction of apoptosis and cell-cycle block at the G2/M phase transition. These results suggest that MNNG interferes with the proliferation of U937 cells, and that a possible mechanism of this interference is arrest at G2/M, which is induced by modulation of cell cycle-regulators.

In terms of regulation of the cell cycle, Cdkks play a most critical role. Two major mechanisms for Cdk regulation are binding with its catalytic subunit cyclin, followed by activation of Cdk/cyclin complexes, and binding with Cdk inhibitors followed by inactivation of Cdk/cyclin complexes [11,33,36]. An alteration in the formation of these complexes could lead to increased cell growth and proliferation, and decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis. A protein kinase, Cdk2 that interacts with cyclin E and cyclin A has been found to play an important role in the regulation of the S and G2 phase. Cdc2 interacts with cyclin B, and activation of the cyclin B/Cdc2 complex is required for transition from the G2 to M phase of the cell cycle. In addition, increased kinase activity induces the phosphorylation of histone H1 and lam in of the nuclear membrane, followed by breakdown of the membrane and chromosome formation, leading the cells to the start of mitosis [24,30,37]. Based on these reports, we investigated the effects of MNNG on the expression of G2/M regulatory factors. The results from the RT-PCR and immunoblotting analyses demonstrated that MNNG did not affect the intracellular protein levels of cyclin A, cyclin B1, Cdk2 and Cdc2 in U937 cells (Fig. 3).
We further examined whether MNNG inhibits the kinase activity of cyclin/Cdk complexes using histone H1 as substrates. The results from the immunocomplex kinase assays using specific antibodies to cyclin B1 and Cdk2 demonstrated that MNNG markedly inhibited both cyclin B1 and Cdk2-associated kinase activities, rather than altering those protein levels (Fig. 4). Taken together, these results suggest that MNNG suppressed cell proliferation by down-regulating the activity of cyclin/Cdk complex kinase and consequently arrested the cell cycle at G2/M in U937 cells. Because we supposed the down-regulation of cyclin/Cdk complex activities by MNNG may be also caused by additional inhibitory mechanism(s), to elucidate this hypothesis we next investigated whether Cdk inhibitors are involved in the MNNG-induced G2/M arrest. This hypothesis was clearly supported by the fact, that MNNG selectively induces the expression of both mRNA and protein levels of Cdk inhibitor p21 (Fig. 5).

It was first reported that Cdk inhibitor p21 is potently transactivated by the tumor suppressor gene product p53, which has further been shown to mediate the induction of cell cycle arrest at the G1 phase by inhibiting Cdk4/Cdk2 activities [14]. In addition to p53, p21 can also be transactivated via a p53-independent pathway in various cell lines stimulated for growth arrest and differentiation. Activation of p21 by vitamin D3 and STAT1 occurs more than 500 base pairs upstream of the MNNG-responsive region [8,28]. In U937 cells, it has been demonstrated that activation of p21 transcription by phorbol esters (TPA) and okaic acid is mediated by the promoter region between 154 and +16 and that Sp1 plays a critical role in this response [5]. In other cancer cells including K562, SW480, and HepG2 cells, the activation of p21 transcription by TPA was mapped to the region between 95 and 121 [4]. Additionally, many studies have shown that Sp1 is a critical factor in regulating transcriptional responses to chemotherapeutic agents such as histone deacetylase inhibitors, alkylphospholipids, vitamin D3, lovastatin etc., [12,19,23,26-28,38] as was observed in this study. Although previous results showed that an increase of wild-type p53 protein level and phosphorylated form of p53 protein accompanied the G2/M arrest caused by MNNG [7,16,18], the present results clearly demonstrated that the induction of p21 by MNNG might be independent on wild-type p53 and Sp1 may play a critical role in MNNG-induced activation of the p21 promoter. Our data also suggested that the down-regulations of cyclin B1 and Cdk2-associated kinase activities are caused by selective induction of p21 expression.

In summary, the present study demonstrates that 1) reduced survival of U937 cells after exposure to MNNG is associated with G2/M phase cell cycle arrest and apoptosis; 2) MNNG can inhibit cell cycle progression at the G2/M phase by decreasing cyclin B1 and Cdk2-associated kinase activity accompanied with a p53-independent induction of the Cdk inhibitor, p21; 3) treatment with MNNG results in the transcriptional activation of p21 through Sp1 binding sites. Taken together, our data show that Sp1 plays an important role in MNNG-mediated G2/M arrest in U937 cells, and provide a basic mechanism for the anti-cancer properties of MNNG in cancer cells.

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초록: N-methyl-N'-nitro-N'-nitrosoguanidine에 의한 인체백혈병세포의 G2/M arrest 유발에서 Cdk inhibitor p21(WIP1/CIP1)의 관련성

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