The cJun N-terminal kinase (JNK) signaling pathway mediates induction of urokinase-type plasminogen activator (uPA) by the alkylating agent MNNG

Maribel Parra, Frederic Lluis, Francesc Miralles, Carme Caelles, and Pura Muñoz-Cánoves

The monofunctional alkylating agent N-methyl-N-nitro-N-nitrosoguanidine (MNNG) is a widespread environmental carcinogen that causes DNA lesions, leading to cell death. However, MNNG can also trigger a cell-protective response by inducing the expression of DNA repair/transcription-related genes. We demonstrate that the urokinase-type plasminogen activator (uPA) gene product, a broad spectrum extracellular protease to which no DNA repair function has been assigned, is transcriptionally induced by MNNG in C2C12 and NIH3T3 cells. This induction required an AP1-enhancer element located at ~2.4 kilobase (kb), because it was abrogated by deletion of this site. MNNG was found to induce the activation of JNK/SAPK and p38 mitogen-activated protein kinases (MAPKs). Accordingly, we attempted to assess the contribution of each of these MNNG-inducible MAPKs to uPA gene induction by this alkylating agent. Coexpression of dominant negative versions of kinases of the JNK pathway, such as catalytically inactive forms of MEK1, MKK7, and JNK, and of cytoplasmic JNK-inhibitor JIP-1, as well as treatment of cells with curcumin (which blocks JNK activation by MNNG), inhibited MNNG-induced uPA transcriptional activity. In contrast, neither dominant negative MKK6 nor SB203580, which specifically inhibit p38 MAP kinase activation, abrogated the MNNG-induced effect. Taken together, our results show that the JNK signaling pathway links external MNNG stimulation and AP1-dependent uPA gene expression, providing the first functional dissection of a transcription-coupled signal transduction pathway for MNNG. (Blood. 2000;96:1415-1424)

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Introduction

Urokinase-type plasminogen activator (uPA) is a secreted serine protease that converts the zymogen plasminogen to plasmin, a trypsin-like serine protease capable of degrading extracellular matrix components and of activating other proteinases (for revision see Irigoyen et al). Analysis of the consequences of loss in uPA function of uPA-deficient mice has confirmed the participation of this protease in physiologic processes such as fibrinolysis and angiogenesis, as well as in pathologic events such as wound healing, inflammation, and tumor invasiveness.

In addition to these proteolytic functions, recent studies have demonstrated mitogenic and chemotactic properties of uPA through interaction with its cell surface high-affinity receptor. Reflecting its wide spectrum of functions, uPA expression is regulated by numerous extracellular stimuli depending on the cell type. uPA gene transcription can be induced by growth factors, phorbol esters, cytokines, cytoskeletal reorganization, and several oncogenes.

We have recently shown that ultraviolet (UV) light irradiation induces the expression of urokinase-type plasminogen activator (uPA) gene in NIH3T3 fibroblasts. uPA was the first protein shown to be inducible in xeroderma pigmentosum cells at much lower UV doses than in parental heterozygotic cells, suggesting that DNA damage might be responsible for this induction. Damage to DNA can be inflicted by a broad spectrum of agents, including UV light, ionizing radiation (IR), and alkylating agents. Recent studies have demonstrated that extracellular uPA activity is inversely related to the cell capacity to repair the DNA lesions induced by alkylation agents such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Monofunctional alkylating agents like MNNG and methyl-methanesulfonate (MMS) are widely distributed environmental mutagens and carcinogens that, on activation, react with DNA and proteins generating adducts. Among the adducts, O6-alkyl guanine (generated by N-alkylation of the DNA base) is the predominant cytotoxic and mutagenic lesion, because of its mispairing properties, which leads eventually to chromosomal aberrations, point mutations, and cell killing. This lesion also appears to be involved in tumor induction, in particular gastric carcinogenesis. However, monofunctional alkylating agents not only cause cell destruction, but also induce the transcription of many genes, including genes coding for transcription factors such as c-fos, c-jun, junB, and junD, for cell cycle regulatory proteins such as p53 and p21, for growth arrest and DNA damage (GADD) proteins and for DNA repair proteins such as O6-methylguanine-DNA-methyltransferase (MGMT) and DNA polymerase β (β-pol).

Recent evidence suggests that the response to DNA-damaging agents may have a protective function other than DNA repair. The main target of genotoxic agents was believed to be chromosomal DNA damage, which in turn would provide the primary signal, triggering the response. However, the rapid UV light activation of Ras, Src, and other molecules located at or near the plasma membrane argues against DNA damage as the primary signal, suggesting that a nuclear signal is not always required for the UV response.
Similarly, genomic DNA has been ruled out as a prerequisite for the induction of JNK activation in response to MMS, because the induction was also detected in enucleated cells.\textsuperscript{48} However, although the first cellular reaction detectable in UV-irradiated cells is the phosphorylation of different cell membrane growth factor receptors at tyrosine residues,\textsuperscript{49} the response to MMS seems to be independent of growth factor receptor activation, suggesting that the primary cellular target of alkylation-driven stimulation is different from that of UV.\textsuperscript{48} Altogether, the signaling cascades induced by alkylating agents appear to be complex. Wherever generated, the alkylating signal, like the UV-induced signal, seems to activate different components of the mitogen-activated protein kinase (MAPK) family, which mediate transcription factor activation (reviewed in Bender et al\textsuperscript{49}). Three MAPK cascades have been shown to transduce stress signals to the nucleus: ERK, JNK/SAPK, and p38. Although JNK and p38 are predominantly involved in the response to stresses and cytokines, ERK is mainly implicated in the regulation of cell growth and differentiation.\textsuperscript{43,51-71} The alkylating agents MMS and MNNG are potent inducers of JNK activation in 293 human cells.\textsuperscript{48} MMS induced p38 phosphorylation in these cells, but did not activate ERK. In contrast to MMS, ethylnitrosonium ethyl nitrosourea (ENU), the most powerful alkylating agent in inducing mutagenesis, failed to induce any of the 3 subgroups of MAPKs, indicating that activation of MAPKs is not a general response to alkylating agents.\textsuperscript{48}

In this study, we show that the monofunctional alkylating agent MNNG is a potent inducer of uPA gene expression in 2 different murine cell lines. Specifically, we have examined the mechanism(s) of MNNG-induced uPA transcription. This induction required an AP1-enhancer element, which is located at -2.4 kilobase (kb) from the uPA transcription start site, and was mediated by the JNK signaling pathway.

**Materials and methods**

**Cell culture**

The murine C2C12 and NIH3T3 cell lines were obtained from the American Type Culture Collection and grown in DMEM containing 10% fetal bovine serum (FBS). For MNNG stimulation, cells were kept in DMEM containing 0.5% FBS for 16 hours. The next day, cells were treated with MNNG (70 \(\mu\)M) for different periods. Alternatively, cells were treated with 20% FBS or UV irradiated at 254 nm (30 J/m\(^2\)). When indicated, cells were pretreated for 30 minutes with 30 \(\mu\)mol/L curcumin, 50 \(\mu\)mol/L SB203580, before induction with MNNG. MNNG and curcumin are from Sigma, and PD98059 and SB203580 from Calbiochem. MNNG was dissolved according to Kaina et al.\textsuperscript{41}

**RNA analysis**

Total RNA was extracted from cells using the commercial Ultraspec RNA isolation system (Biotecx) based on the Chomczynski method.\textsuperscript{22} Northern analysis using uPA and GAPDH probes was described previously.\textsuperscript{8}

**Plasmids**

The p-8.2Luc, a murine uPA-promoter luciferase reporter plasmid (kindly provided by Dr Y. Nagamine), contains 8.2 kb of murine uPA promoter (reviewed in Bender et al\textsuperscript{49}). The p-8.2Luc, lacking the AP1 enhancer element, are described elsewhere.\textsuperscript{13,23,72} Expression vectors pSRE-MEKK1(K432M), pCDNAII-MKK6b(A), pCDNAIII-HP-1, pCDNAIII-SEK1/MKK4(KR), and pCDNAII-MKK7(A) were kindly provided by Drs M. Karin, J. Han, R. Davis, and E. Nishida.\textsuperscript{57,63-77}

**Western blotting**

Cells were cultured in 0.5% FBS and, at the indicated time points after treatment, whole cell extracts (WCE) were prepared as described in Miralles et al.\textsuperscript{21} Total JNK1 and ERK2 were detected in 30 \(\mu\)g WCE by immunoblotting using specific antibodies at 1:1000 dilution. JNK1 and ERK antibodies are from Santa Cruz Biotechnology (sc-474 and sc-154, respectively). Alternatively, phosphorylated p38 was detected using an antiphospho p38 antibody (New England Biolabs No 921S). Immunoblots were developed using ECL detection system (Amersham).

**Protein kinase assays**

JNK and ERK were immunoprecipitated from WCE with anti-JNK1 and anti-ERK2 antibodies, respectively, and immunocomplexes were recovered with protein A-Sepharose and washed, as described previously.\textsuperscript{23} Phosphorylation reactions were performed in a 30-\(\mu\)L volume containing kinase buffer supplemented with 20 \(\mu\)mol/L ATP, 0.0185 MBq (0.5 \(\mu\)Ci) \(\gamma\)-[\(\beta\)]ATP and 1 \(\mu\)g GST-cJun\textsuperscript{1-79} or myelin basic protein (MBP) as substrates for JNK and ERK assays, respectively, at 30°C for 30 minutes. Reactions were stopped by the addition of 4 \(\times\) Laemmli sample buffer and resolved by 10% or 12% SDS-PAGE for JNK or ERK assays, respectively.

**Transfections assays**

2.5 \(\times\) 10\(^4\) cells were cotransfected using the transfection reagent DOTAP (Boehringer Mannheim) with 300 ng of uPA-luciferase plasmid and 50 ng of RSV-\(\beta\)Gal, as internal control. After transfection, cells were cultured in DMEM containing 0.5% FBS for 16 hours before MNNG stimulation (70 \(\mu\)mol/L), and reporter activities were analyzed after 8 hours. When indicated, cells were cotransfected with 150 ng of reporter plasmid and 150 ng of expression plasmids or empty vector alone, together with 50 ng of internal control. Inhibition of MEK and p38 kinase was performed by pretreating transfected cells with 50 \(\mu\)mol/L and 10 \(\mu\)mol/L SB203580, respectively, for 30 minutes before MNNG treatment. Alternatively, transfected cells were pretreated with 30 \(\mu\)mol/L curcumin for 30 minutes before an 8-hour period of incubation with MNNG. Firefly luciferase activities were standardized for \(\beta\)-galactosidase activity, used as internal control. All transfection/reporter assays were repeated at least 3 times, showing less than 25% variability. A Student \(t\) test was used to validate the results.

C2C12 cell lines containing stables transfected the different uPA promoter-luciferase plasmids have recently been characterized.\textsuperscript{72}

**Electrophoretic mobility shift assays**

Nuclear extracts were obtained from C2C12 cells before and after MNNG treatment. The extraction of nuclear proteins was performed as described by Miralles et al.\textsuperscript{23} For electrophoretic mobility shift assays (EMSAs), 5 \(\mu\)g of nuclear extracts was incubated in 50 mmol/L Tris-HCl pH 7.9, 12.5 mmol/L MgCl\(_2\), 1 mmol/L EDTA, 1 mmol/L DTT, 20% glycerol, 0.5 mmol/L PMSF, and 2 \(\mu\)g of poly dI-dC for 10 minutes at room temperature to titrate out nonspecific binding before the addition of 15 000-20 000 cpm-labeled oligonucleotide and the reaction was further incubated for 20 minutes. When unlabeled competing oligonucleotides were added, nuclear extracts were preincubated for 30 minutes at room temperature before the addition of the labeled probe. Samples were loaded on a prerun 5% polyacrylamide gel (29:1 in 0.25 \(\times\) TBE) and electrophoresed at 200 V. Gels were dried and autoradiographed at \(-80^\circ\) C. The sequences of the sense strands of the oligonucleotides used in EMSAs are as follows:

\texttt{AP1}\textsubscript{p\_4}, \texttt{5\_GAGGGAATTGAGGATCATCTGTGCCTCG-3}'
\texttt{AP1}\textsubscript{p\_5}, \texttt{5\_GAGGGAATTGAGGATCATCTGTGCCTCG-3}'
\texttt{IgKB}, \texttt{5\_CAAGGGGACCTTCCGAG-3}'

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Results

MNNG induces murine uPA messenger RNA (mRNA) expression. We have previously shown that uPA expression is induced during the UV response. To extend this observation, we analyzed whether other environmental mutagenic agents, such as the mono-functional alkylating agent MNNG, modulate the expression of the uPA gene. As shown in Figure 1A, treatment of C2C12 and NIH3T3 murine cell lines with MNNG increases the expression of a 2.7-kb transcript corresponding to murine uPA mRNA, with respect to untreated cells. uPA mRNA induction was not due to an unspecific up-regulation of RNA synthesis, because MNNG did not significantly modify the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in either cell line. The increase in uPA gene induction was time-dependent, being first observed after 30 minutes after stimulation in C2C12 cells and reaching its maximum 2 hours after MNNG treatment; uPA mRNA induction in these cells decreased 4 hours after treatment, returning to basal levels after 8 hours (Figure 1A, top). Induction of uPA mRNA in NIH3T3 cells was maximal after 4 hours stimulation with MNNG, and was undetectable by 8 hours after treatment (Figure 1A, bottom). To gain an insight into the mechanisms leading to increased uPA mRNA expression in MNNG-treated cells, we studied the effects of RNA and protein synthesis inhibitors on the uPA transcript level in cells that were stimulated with the alkylating agent. The protein synthesis inhibitor cycloheximide did not inhibit uPA mRNA induction by MNNG in either cell type (Figure 1B, and data not shown); moreover, cells treated only with cycloheximide expressed higher levels of uPA transcripts, suggesting that MNNG stimulation of uPA mRNA did not require de novo protein synthesis. In contrast, uPA mRNA induction by MNNG was abrogated in cells treated with the RNA synthesis inhibitor actinomycin D (Figure 1B). These data suggested that increased uPA transcription, rather than message stabilization, was the mechanism responsible for the MNNG-induced effect.

An AP1-enhancer element is involved in uPA promoter induction by MNNG

We next examined the effect of MNNG on the activity of the murine uPA gene promoter. A murine uPA genomic fragment (−8.2 kb to +398 base pairs [bp]), ligated upstream the luciferase reporter gene, p−8.2Luc, was assessed for luciferase activity after transient transfection in C2C12 and NIH3T3 cells. Comparison of luciferase activities generated by p−8.2Luc, between unstimulated and MNNG-stimulated cells, showed that the uPA promoter activity increased an average 4-fold after MNNG treatment in both cell types, whereas activity of p−0.035Luc (a plasmid including only 35-bp of the uPA minimal promoter sequence) was unaffected (Figure 2A), indicating that the murine uPA promoter contains MNNG-responsive sequences that might account, at least in part, for the MNNG-mediated induction of uPA in these cells. To begin identifying the main regions involved in the uPA transcriptional response to MNNG, different murine uPA promoter-deletion luciferase constructs, generated from the full-length −8.2-kb promoter plasmid, were stably transfected into C2C12 cells, and the corresponding uPA promoter-luciferase (uPA-Luc)–containing cell lines were analyzed for luciferase inducibility after treatment with MNNG. As shown in Figure 2B, deletion of 1.6 and 3.3 kb from the −8.2-kb construct (generating plasmids p−6.6Luc and p−4.9Luc, respectively) did not alter the luciferase induction of the full-length −8.2-kb plasmid (P > .05), suggesting that these upstream promoter regions were irrelevant for uPA transcriptional induction by MNNG in the uPA-Luc–containing cell lines. However, although C2C12 cells containing p−4.9Luc retained full MNNG-induced luciferase activity, C2C12 cells transfected with p−2.0Luc, a plasmid containing 2.0 kb of the murine uPA minimal promoter,
showed no luciferase inducibility ($P < .01$) (Figure 2B), clearly indicating the location within this region of cis-element(s) relevant for MNNG-induced uPA transcription. As expected (according to transient transfection results shown in Figure 2A), cells stably transfected with p-0.035Luc showed no luciferase induction after stimulation with MNNG (Figure 2B).

The uPA promoter contains an AP1-enhancer element, located at −2.4 kb, known to respond to several extracellular stimuli (reviewed in Besser et al 1990). To assess whether this element might also be responsible for MNNG stimulation, we analyzed the transcriptional inducibility of C2C12 cell lines expressing uPA-Luc constructs containing or lacking the AP1-enhancer site.72 Figure 2C shows that deletion of the AP1 enhancer element in p-8.2Luc, p-8.2ΔAP1Luc totally abrogated luciferase induction by MNNG in C2C12 cells. Similar results were obtained with additional uPA-Luc-C2C12 cell lines containing a specific deletion of the AP1-enhancer element: p-6.6ΔAP1Luc and p-4.9ΔAP1Luc-containing cell lines showed no luciferase inducibility by MNNG (data not shown). These results demonstrated the requirement of the AP1-enhancer for uPA transcriptional activation by MNNG. Moreover, the uPA AP1-enhancer showed increased AP1 binding activity after treatment of the cells with MNNG (Figure 2D). The uPA AP1-enhancer is composed of 2 phorbol ester responsive elements (TRE): an upstream octameric AP1A site (5′-TRE) and a downstream canonical AP1B site (3′-TRE), separated by an intervening element (COM). As shown in Figure 2D, both AP1A and AP1B sequences of the uPA AP1-enhancer bound a major nuclear protein complex, whose intensity increased after MNNG treatment of C2C12 cells, as assessed by EMSA (left). When each oligonucleotide was used as an unlabeled competitor, the formation of the corresponding DNA-protein complex was prevented; in contrast, excess of an unrelated competitor (the kB site of the Igκ enhancer, IgkB) sequence did not affect the formation of either complex (Figure 2D, right), demonstrating the specificity of the DNA-protein interaction.

**Induction of MAP kinases by MNNG in C2C12 and NIH3T3 cells**

The alkyllating agent MMS has been shown to promote the induction of JNK and p38 activities in human 293 cells.66 MNNG could also induce JNK activity in these cells. However, JNK activation is not a general response to alkylating drugs, because alkyllating agents such as ENU cannot exert this effect. To determine the potential activation of the 3 main classes of MAPKs (JNK, ERK and p38) by MNNG in C2C12 and NIH3T3 murine cell lines, we measured the activities of JNK, ERK and p38, respectively, in response to MNNG treatment (Figure 3). Accordingly,
immune complex kinase assays with C2C12 and NIH3T3 cell extracts, using an antibody against JNK1 and GST-cJun as substrate, were performed to determine JNK activation by MNNG in these cells. MNNG induced a very strong activation of JNK activity, peaking 1 hour after stimulation, remaining high after 2 hours, and returning to basal levels 4 hours after treatment in C2C12 cells (Figure 3A, left). MNNG was also a very potent inducer of JNK activity in NIH3T3 cells, reaching maximal levels between 30 minutes and 1 hour after stimulation, decreasing after 2 hours, and returning to basal levels 4 hours after treatment (Figure 3A, right). Similarly, we measured the activation of ERK using an antibody against ERK2 and myelin basic protein (MBP) as a substrate. As shown in Figure 3B, no activation of ERK2 was observed after MNNG treatment (up to 2 hours) in C2C12 cells, whereas ERK2 was potently activated in these cells within 6 minutes (0.1 hour) after serum (20% FBS) treatment. In C2C12 cells, no ERK activation was observed after MNNG treatment of NIH3T3 cells (data not shown). Finally, activation of p38 by MNNG in C2C12 cells was assessed by Western blotting using an antibody specific for phospho-p38. As shown in Figure 3C, phosphorylation of p38 by MNNG was detected within 30 minutes (0.1 hour) after serum (20% FBS) treatment. As in C2C12 cells, MNNG induced a very weak phosphorylation of p38 in NIH3T3 cells (data not shown). Altogether, these results suggested that JNK is the most potently activated MAPK in response to MNNG in C2C12 and NIH3T3 cells, whereas p38, but not ERK, was weakly activated by MNNG in these cells.

Curcumin blocks JNK activation by MNNG

PD98059 and SB203580 are specific inhibitors of ERK and p38 activation, respectively. However, no synthetic inhibitor of JNK activation has yet been identified. The dietary pigment curcumin is a potent inhibitor of JNK activation by various agonists, including PMA plus ionomycin, anysosycin, UV-C, and TNFα. To test whether curcumin could also inhibit JNK activation in response to MNNG, C2C12 cells were pretreated with curcumin before their stimulation with MNNG, and cell extracts were immunoprecipitated with an antibody against JNK1, and the enzymatic activity assessed using GST-cJun as the substrate. As shown in Figure 4, activation of JNK activity after a 1-hour treatment with MNNG was completely abrogated by curcumin pretreatment, thus providing a useful tool for blocking JNK activation by this alkylating agent. As expected, neither 10 μmol/L SB203580 nor 50 μmol/L PD98059 affected JNK activation by MNNG, whereas these compounds inhibited activation of p38 by UV-C irradiation and of ERK2 by 100 nmol/L TPA, respectively, in NIH3T3 and C2C12 cells (results not shown).

Curcumin abrogates induction of AP1 binding, uPA transcription, and uPA mRNA expression by MNNG

Once we had shown that both JNK and p38 MAPKs are induced in response to MNNG in C2C12 cells, we analyzed whether any of these MAPK signaling pathways were responsible for the induction of uPA gene expression by MNNG. We studied the effect of JNK and p38 inhibitors on uPA expression at different levels (Figure 5). As shown by Northern blotting, uPA mRNA expression was induced in C2C12 cells after a 2-hour treatment with MNNG. Curcumin pretreatment of C2C12 cells resulted in a complete...
inhibition of uPA mRNA induction by MNNG (Figure 5A); in contrast, pretreatment of cells with p38 inhibitor SB203580 did not alter this induction and, as expected, ERK inhibitor PD98059 had no effect on uPA induction by MNNG (Figure 5A, right). These data suggested that uPA gene induction by MNNG most likely occurred via the JNK signaling pathway.

We next examined whether these MAPK inhibitors modify the MNNG-transcriptional response of uPA-Luc-containing C2C12 cell lines. As shown in Figure 5B, MNNG induced luciferase activity from the p-8.2Luc-C2C12 cell line an average of 4-fold. Although neither PD98059 nor SB203580 had any significant effect on uPA promoter induction by MNNG, curcumin fully abrogated uPA transcriptional induction in response to MNNG. Moreover, pretreatment of C2C12 cells with curcumin decreased AP1-binding activity to the uPA 3'-TRE (AP1B) after stimulation with MNNG (Figure 5C). In summary, curcumin specifically inhibits uPA induction by MNNG at 3 different levels: (1) AP1-binding to the uPA enhancer element, (2) uPA transcriptional activity, and (3) uPA mRNA expression.

**Involvement of the JNK signaling pathway in uPA transcriptional induction by MNNG**

Because only curcumin seemed to abrogate uPA induction by MNNG, we hypothesized that the JNK signaling cascade was the intracellular mediator of this effect. To determine the specific involvement of the JNK pathway in uPA gene induction by MNNG, we determined uPA transcriptional activation by this agent in the absence or presence of specific MAPK inhibitors. MEKK1(K432M), a dominant negative form of MEKK1, was overexpressed in transient cotransfection experiments with p-6.6Luc in C2C12 cells, with or without MNNG. As shown in Figure 6, the uPA promoter activity induced by MNNG was strongly reduced by MEKK1(K432M), indicating the requirement of the MEKK1-JNK pathway for uPA induction by MNNG.
the catalytically inactive mutant form of MEKK1. Furthermore, overexpression of JIP-1, a cytoplasmic protein known to cause retention of JNK in the cytoplasm and subsequent inhibition of the JNK pathway,57 and to act as a scaffold protein for JNK signaling,58 also down-regulated MNG-induced uPA promoter activity in these cells. In addition, the expression of MKK7(A), a dominant negative form of MKK7, a specific activator of JNK, but not of p38, in response to TNFα and UV,59 abrogated uPA transcriptional induction by MNG. A similar inhibitory effect on uPA promoter induction by MNG was caused by overexpression of SEK1(KR), a dominant negative form of SEK1 (JNK kinase, also known as MKK4) (Figure 6). These results, together with those showing the inhibitory effect of curcumin on uPA promoter-luciferase stimulation (Figure 5B), indicated that the transcriptional induction of the uPA gene by MNG was mediated, at least in part, by the MEKK1/JNK pathway. In agreement with the results shown in Figure 5B, SB203580, a specific inhibitor of p38 isoforms (p38α and p38β)60,61 did not block the MNG-induced activation of the −8.2-kb promoter-containing C2C12 cell line. However, because 2 additional p38 isoforms (p38γ and p38δ)60,61 are activated by stresses such as UV but insensitive to SB203580,60,61 the potential involvement of these latter kinases in uPA induction by MNG was assessed. Accordingly, MKK6b(A), a dominant negative form of MKK6b, which diminishes the activation of all p38 isoforms,60,61 was cotransfected together with p-6.6Luc in C2C12 cells. As shown in Figure 6, the uPA promoter activity induced by MNG was not suppressed by the catalytically inactive mutant form of MKK6b. Taken together, these results indicated that the JNK pathway was directly involved in the transcriptional activation of the murine uPA gene by MNG in C2C12 cells, via its AP1-enhancer element.

Discussion

Genotoxic agents like UV light irradiation and monofunctional alkylating carcinogens trigger a rapid, highly regulated adaptive response, known as the cellular stress response, which involves coordinate control of multiple signal transduction pathways, leading to the induction of many genes. The gene inductive response to UV has been analyzed extensively, and it is known to promote transcription of genes coding for transcription factors, growth factors, viral proteins, and proteases (reviewed in Bender et al50). However, less is known about the inductive response to alkylating agents such as MMS and MNG. These agents induce the early expression of several proto-oncogenes including c-fos, c-jun, junB, and junD, although to a different extent.55 They also induce the level of cell cycle regulatory/tumor suppressor proteins such as p53, p21, and adenomatous polyposis coli (APC),36,38,82 and of DNA repair proteins such as O6-methylguanine-DNA methyltransferase (MGMT) and β-pol,41,42 as well as growth arrest and DNA damage-inducible (GADD) proteins.79,80 However, no additional cellular targets of alkylating agents, other than those related with gene transcription or DNA repair processes, have been identified in mammalian cells. Interestingly, in 1986, Brdar reported the induction of plasminogen activator (PA) activity by alkylating agents in a DNA repair-defective human glioblastoma cell strain,81 although whether this enzyme was a direct target for alkylating agents, and which were the mechanisms responsible for PA induction remained to be solved. Here we show that the uPA (urokinase-type plasminogen activator) gene product, a broad-spectrum extracellular protease, can be induced by the alkylating agent MNG via the JNK signaling pathway. In particular, we found that an AP1-enhancer element, which is conserved in murine, porcine, and human uPA promoters, is required for the induction by MNG, because the deletion of this element abrogated the induction. Moreover, this is the first functional dissection of a transcription-coupled signal transduction pathway for MNG induction.

The cellular response to genotoxic agents is complex. UV irradiation activates different MAPKs (ERK, JNK, and p38) as well as NFκB in numerous cell systems (reviewed in Bender et al50). Induction of JNK and p38 activation by MMS has also been shown in the human embryonic cell line 293,48 although no attempts were made to relate these events to the expression of endogenous genes. We have recently demonstrated that UV irradiation induced uPA gene expression in mouse fibroblasts.53 On the basis of this observation, we hypothesized that other genotoxic agents, in particular, alkylating agents, might also induce uPA expression during the gene inductive response. We have found that MNG induces uPA mRNA expression in C2C12 and NIH3T3 murine cell lines. However, the time course of uPA mRNA accumulation was different with MNG and UV; UV induction peaked at 24 hours, lasting up to 48 hours, whereas MNG induction occurred much earlier, showing a maximal increase 2 to 3 hours after treatment. Induction by MNG did not require ongoing protein synthesis, indicating that MNG activates preexisting components of a signaling pathway. Inhibition of protein synthesis by itself enhanced the expression of uPA mRNA, which may be due to suppression of a protein with a short half-life involved either in the degradation of uPA mRNA or in repression of uPA gene transcription. Moreover, uPA promoter induction by MNG was completely blocked by inhibitors of cytoplasmic signaling cascades (Figures 4 and 5), which originate at or near the plasma membrane,43 suggesting that the uPA gene could be activated, at least in part, by a cytoplasm-transduced signal. These results are consistent with recent reports showing that the activation of JNK by MMS, like the response to UV light, may not be mediated by DNA damage per se; in fact, the MMS response is independent of a nuclear signal, because JNK induction can be observed in enucleated cells.48 We have shown that JNK and p38, but not ERK, MAP kinases are activated in response to MNG treatment of C2C12 and NIH3T3 murine cell lines. However, JNK is more potently activated than p38 by MNG. The induction kinetics of JNK (or p38) activation in response to MNG are slower than in response to UV-C irradiation in C2C12 and NIH3T3 cells, because the peak of JNK activity in response to the former stress occurs around 1 hour after stimulation (Figure 3A), whereas this activity is maximal 15 minutes after UV-C irradiation.53 In addition, the MNG-induced activation of JNK in both C2C12 and NIH3T3 cells was more transient than in 293 cells; whereas in C2C12 and NIH3T3 cells no JNK activity could be detected 4 hours after MNG treatment (Figure 3A), JNK activation levels were still high in 293 cells at the same time after treatment.48

Promoter deletion analysis revealed that the murine AP1-enhancer element located at −2.4 kb was required for uPA promoter induction by MNG, because its deletion abrogated the induction. Transient transfection assays with coexpression of specific inhibitors for proteins involved in distinct MAPK signaling pathways suggested that JNK, but not p38, is involved in MNG-dependent uPA gene induction. Expression of MEKK1 results in efficient JNK activation without a significant increase in ERK1/2 or p38 activities.54,76 Our results show that overexpression of
MEKK1(K432M), a catalytically inactive form of MEKK1, abrogated
the MNNG-induced up-regulation of the murine uPA pro-
moter in C2C12 cells. The involvement of JNK in MNNG-induced
uPA transcription was further shown by the inhibitory effect caused
by overexpression of JIP-1, a cytoplasmic inhibitor of JNK,57
which also functions as a scaffold protein for the JNK signaling
pathway.58 Similarly, overexpression of dominant mutant versions
of SEK1 (M KK4), which activates both JNK and p38,55,56 or
MKK7, a MAPK kinase isofrom specific for JNK activation by
TNFα, as well as by environmental stresses,57 down-regulated uPA
transcriptional induction by MNNG. Moreover, we have previ-
ously shown that constitutively active MEKK1 activates uPA gene
transcription in NIH3T3 cells, in the absence of MNNG stimula-
tion, suggesting that the uPA gene is a target for this MAPK
pathway.23

Reports from over a decade ago indicate that the alkyllating
agents mechlorethamine and MNNG could induce the production
of plasminogen activator in U-87MG cells, an alkylation repair-
deficient (Mer−) human glioblastoma strain, at much higher levels
than in alkylation repair-proficient (Mer+) U-178MG cells.85 It
was concluded that plasminogen activator induction in alkylation
repair-deficient human cells is caused by unrepaird DNA damage
and may represent an eukaryotic SOS-like function. In fact, most
of the MNNG-inducible genes identified in mammalian cells appear
to be involved in DNA repair in a way similar to that of the bacterial
SOS response. However, several reports have suggested that the
mammalian stress response to genotoxic agents was involved in a
protective function other than DNA repair. In particular, the
inhibition of the UV response by tyrosine kinase inhibitors has
confirmed its protective role.43 Furthermore, c-fos(−/−) cells and
c-jun(−/−) cells are hypersensitive to UV,84 suggesting that the
UV-inducible cJun and cFos are essential components of the
cellular defense mechanisms against the cytotoxic effects of UV.
Similarly, cells deficient in cFos are also hypersensitive to a broad
spectrum of DNA-damaging carcinogens, including MMS and
MNNG, showing that cFos/AP1 plays a decisive role in cellular
defense against genotoxic agents.44 The results shown in this study
indicate that the alkyllating carcinogen MNNG induces the expres-
sion of a broad spectrum extracellular protease, uPA, with no DNA
repair properties. Moreover, we demonstrated that the induction of
uPA gene expression by MNNG occurs in the first hours after
treatment and is mediated by a cytoplasmic signaling pathway,
independent of DNA damage. These results contrast with previous
reports suggesting that the induction of extracellular plasminogen
activator activity, whose peak occurred 36 to 48 hours after
alkylation treatment, is inversely related to the cell capacity to
repair the DNA lesions induced by alkyllating agents.85,86 However,
the mechanism involved in this late DNA repair-dependent uPA
induction by MNNG remains to be identified. It could be specu-
lated that in DNA repair-proficient cells (this study), the early
induction of uPA gene expression in response to MNNG is
originated and mediated by cytoplasmic events, independently of
DNA damage. In contrast, in DNA repair-deficient cells, the
unrepaired DNA damage caused by MNNG might also induce uPA
gene expression through a mechanism that has yet to be explained.
The physiological significance of the uPA induction by genotoxic
agents is unknown. Exposure of cells to MNNG and most other
DNA-damaging agents results in damage of biomembranes, pro-
teins and nucleic acids, either directly or after oxidative stress.59
A simple protective mechanism against damage to such components
would consist of replacing them with newly synthesized ones. As
already mentioned, exposure of cells to DNA-damaging agents can
increase DNA repair capacity and activate cell-cycle checkpoints,
but such exposures may also induce enzymes that metabolize
toxins to facilitate their elimination from the organism or may
activate programmed cell death (apoptosis) to eliminate highly
damaged cells. Accordingly, the increased expression of the
proteolytic enzyme uPA after MNNG treatment may facilitate the
clearance of the damaged cell components. This protease, in
conjunction with other extracellular proteinases, has a function in
the degradation of the extracellular matrix, in conditions like
wound healing, inflammation, and cancer. Recent work with
uPA-deficient mice has demonstrated that uPA is required for the
inflammatory response to Cryptococcus neoformans, because lack
of uPA resulted in inadequate cellular recruitment, uncontrolled
infection and death.8 In contrast, using uPA-mutant mice, it has
been shown that the PA system is a causal component of poly-
oma middle T-induced vascular tumor formation, implying a role
for uPA in vascular oncogenesis.86 Taken together, these studies
suggest that uPA may have both beneficial and deleterious roles
during the response to different pathologic situations. The
functional significance of uPA induction by alkyllating agents
remains to be determined. At present, uPA-deficient mice are
available. The sensitivity of uPA−/− cells to MNNG treatment is
expected to clarify the role of this protease in the cellular response
to alkyllating agents.

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