Overexpression of Metallothionein-II Sensitizes Rodent Cells to Apoptosis Induced by DNA Cross-linking Agent through Inhibition of NF-κB Activation*

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DNA cross-linking agents such as mitomycin C (MMC) and cisplatin are used as chemotherapeutic agents in cancer treatment. However, the molecular mechanism underlying their antitumor activity is not entirely clear. Critical steps in cytotoxicity toward cross-linking agents can involve DNA repair efficiency, inhibition of replication, cell-cycle checkpoints, regulation, and induction of apoptosis. The complexity of the mechanisms of the mammalian cell defense against cross-linking agents is reflected by the existence of many complementation groups identified in rodent cells that are specifically sensitive to MMC. We recently showed that increased induction of apoptosis contributes to the MMC sensitivity of the group represented by the V-H4 hamster mutant cell line. In this study, through the analyses of a subtractive library, we discovered that sensitive V-H4 cells display a 40-fold increase of steady-state expression of metallothionein II (MT-II) mRNA compared with resistant parental V79 cells. Down-regulation of MT-II by antisense oligonucleotides partially restores MMC resistance in V-H4 cells, indicating that MT-II overexpression is directly involved in MMC hypersensitivity of these cells. MTs have been reported to regulate the activation of NF-κB, one of the key proteins that modulates the apoptotic response. Here we found that NF-κB activation by MMC is impaired in V-H4 cells and is partially restored following down-regulation of MT-II by antisense oligonucleotides. All these data suggest that the overexpression of MT-II in V-H4 cells impairs NF-κB activation by MMC, resulting in decreased cell survival and enhanced induction of apoptosis.

DNA cross-linking agents constitute a set of pharmacological molecules used as single agents or in combination in the treatment of a wide variety of malignant tumors (1). Among them, mitomycin C (MMC)† is more used particularly for the treatment of adenocarcinomas, non-small cell lung cancer, some head and neck cancers, and in chronic myelogenous leukemia (2). MMC is activated in vivo to an alkylating agent by a reductive activation cascade and forms monoadducts and interstrand or intrastrand cross-links on DNA, preferentially at the N2 position of guanine (3). The molecular mechanism underlying the MMC antitumor activity is not fully understood. To elucidate the mechanisms of the mammalian cell defense against cross-linking agents, mutants specifically sensitive to MMC have been isolated in rodent cells (4). The genetic and biochemical complexity of these processes is reflected by the existence of at least eight complementation groups identified among rodent cell mutants defective in the response to MMC treatment (5, 6).

V-H4 cell line, a representative of one of these complementation group, was isolated from V79 Chinese hamster cells (7). V-H4 mutant cells exhibit increased sensitivity toward cross-linking agents such as MMC (~30-fold more sensitive than the wild-type V79 cells) and cisplatin (~10-fold more sensitive), but they are not hypersensitive to UV light, H2O2, or x-rays (7). The V-H4 cell response to this panel of genotoxic agents suggests that the defective protein or pathway in these cells is specific to cross-linking agents. However, the molecular defect responsible for the sensitivity of this mutant cell line to MMC remains to be determined. Critical steps in cytotoxicity toward cross-linking agents can involve DNA repair efficiency (8), inhibition of replication (9), cell-cycle checkpoints regulation, and induction of programmed cell death (10). We showed previously that neither a defect in nucleotide excision repair of DNA interstrand cross-links (11) nor a defective G2 phase checkpoint contributes to the differential sensitivity of V-H4 mutant toward MMC (12). In contrast, our findings demonstrated that sensitive V-H4 cells undergo greater levels of apoptosis than resistant parental cells following both equimolar and equitoxic MMC treatment (12). This differential apoptotic response is specific for the cross-linking agent MMC and is p53-independent, because p53 sequence is mutated in V-H4 cells (12) as well as in V79 parental cells (13). Our previous results suggested then that control of the apoptotic process is altered in V-H4 mutant cells. Defective gene(s) in these cells could function in the regulation of an apoptotic pathway triggered by MMC-induced damages and independent of p53-mediated transcription. Product(s) of this gene(s) could interfere at different levels of this process, such as detection of the MMC adduct on the DNA or of an intermediate of lesion repair, presence of reactive oxygen species produced during MMC detoxification, or even being directly implicated in the transduction cascade of the apoptotic signal initiated by MMC.

To further characterize the molecular defect in V-H4 cell line, we sought to identify gene(s) involved in MMC hypersensitivity of these cells. In this report, we compared mRNA expression pattern of V-H4 mutant cell line with that of V79 parental cell line. Using a suppression subtractive hybridization methodology (14), we found a steady-state overexpression

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§ The abbreviations used are: MMC, mitomycin C; MT-II, metallothionein-II; TNF, tumor necrosis factor; CPT, camptothecin; NF, nuclear factor; EMSA, electrophoretic mobility shift assay.
Cell survival was expressed relative to the number of colonies obtained without drug. Colony-forming efficiencies of V79 and V-H4 cells were routinely 80 and 65%, respectively.

MT-II Down-regulation Assay—Two 26-base oligonucleotides were synthesized: an antisense sequence (5'-GGCGGGGAGCCGAAAGC-3') and a sense sequence (5'-CCCCCACTGCTCTGGCCCCGCGAAAGC-3'), corresponding to the region downstream of the ATG translational start site of human MT-II mRNA sequence between bases 7 and 24 (18). The underlined 8 base-length sequence corresponds to a mini-hairpin structure added to the 3'-end of the oligonucleotides, conferring them a stable structure resistant to 3'-exonuclease degradation (18). Bacteria essentially growing cells were incubated in 0.1 M of poly(dI-dC), poly(dI-dC)-end of the oligonucleotides, containing 5–10 μM oligonucleotide and subjected to electrophoresis (1.4 kV/cm, 1 mHz, 10 impulsions of 1 ms). 10 min later, cells were resuspended in Ham's F-10 medium supplemented with 15% heat-inactivated newborn calf serum and seeded at various dilutions. Efficiency of the antisense oligonucleotide in down-regulating MT-II protein translation was checked by decreased sensitivity to cadmium using a colony-forming growth assay 24 h after electrophoresis using with 10 μM oligonucleotide. The effect of MT-II translation down-regulation on MMC sensitivity was assessed 15 h after electrophoresis using with 5 μM antisense oligonucleotide.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared. 0.5 × 10⁶ C. Bacteria were resuspended in 400 μl of buffer A (10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol), supplemented by Complete™ protease inhibitors (Roche Molecular Biochemicals), allowed to swell on ice for 10 min, vortexed for 10 s, and centrifuged for 1 min at 10,000 × g. Pellets were resuspended in 90 μl of cold buffer C (20 mM Hepes-KOH, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol), supplemented by Complete™ protease inhibitors and incubated on ice for 20 min for high salt extraction. Supernatants containing DNA-binding proteins were obtained after the removal of cellular debris by centrifugation for 2 min at 10,000 × g. Nuclear extracts (10 μg) were incubated with a 32P-labeled double-stranded oligonucleotide (5'-AGTTAGCCGAGGCAAGGAAAAGGC-3') containing the NF-κB consensus sequence (underlined) and 1 μg of poly(dI-dC) (Sigma) in binding buffer (10 mM Hepes-KOH, pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 0.2 mM dithiothreitol) for 30 min at 30 °C. DNA probes were prepared by end-labeling both strands of the oligonucleotide using [γ-32P]ATP (Amersham Biosciences, Inc.) and T4 polynucleotide kinase (Biolabs). A 100-fold excess of non-radioactive probe was used as a control to specifically compete for binding. A double-stranded mutated oligonucleotide was also used to examine the specificity of binding of NF-κB to the DNA (data not shown). Reaction mixtures were loaded onto a 4% non-denaturing acrylamide gel. Gels were run in 0.5% Tris acetate buffer-running buffer at 110 V for 1 h, dried, and exposed to PhosphorImager screen.

RESULTS

MT-II Gene Is Overexpressed in V-H4 Mutant Cells—To reveal a differential gene expression that might be involved in MMC hypersensitivity of V-H4 mutant cells, we compared the steady-state mRNA expression between parental V79 and V-H4 mutant cell lines. Using suppression subtractive hybridization, we isolated a clone containing a 349-bp cDNA corresponding to the complete sequence of hamster metallothionein-II mRNA. Overexpression of MT-II mRNA in V-H4 cells was confirmed by Northern blot analysis after hybridization of total RNA to the probe corresponding to MT-II cDNA as shown on the representative blot in Fig. 1. A 40-fold increase of steady-state MT-II gene expression was found in mutant V-H4 cells compared with the parental cell line V79 (Fig. 1, lanes V79). Similar results were obtained with independent RNA extractions.

MT-II is induced by metals and various agents such as growth factors, onecogene products, and oxidants (21). We investigated whether MT-II mRNA levels can be increased by MMC treatment. V79 and V-H4 cells were treated with 500 ng/ml MMC for 1 h, and MT-II expression was quantified at various times after drug treatment. Our results showed that MT-II mRNA is highly induced as soon as 30 min after treat-
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**Fig. 1.** Differential expression of the MT-II mRNA in resistant V79 and sensitive V-H4 cells. V79 and V-H4 cells were treated with 500 ng/ml MMC for 1 h or left untreated (NT). Total RNA was extracted following drug treatment after the indicated incubation periods. 15 μg of RNA were subjected to Northern blot analysis using a 32P-labeled probe corresponding to the Chinese hamster MT-II cDNA. Membranes were subsequently stripped and rehybridized with a human β-actin probe to standardize for RNA amounts. RNA levels were measured using a PhosphorImager and the ImageQuant™ software (Molecular Dynamics, Inc.).

**Fig. 2.** V-H4 mutant cells are more resistant to CdCl2 toxicity. V79 and V-H4 cells were treated at the indicated concentrations of CdCl2 for 2 h. Cell survival was determined by clonogenic assay 8 days after the treatment. The percent cell survival of V79 and V-H4 cells is shown as a function of CdCl2 dose. Data are derived from three independent experiments done in duplicate for each CdCl2 concentration and are presented as mean values.

MT-II Gene Overexpression Protects V-H4 Cells Against CdCl2 Toxicity—We then examined whether MT-II mRNA overexpression in V-H4 cells was associated with increased MT-II protein levels. It has been shown that increases in mRNA for MT correlate well with the induction of MT protein (22). Unfortunately, MT levels could not be determined in our cell lines by immunoblotting technique or radioimmunoassay as described previously (23), because no available antibody directed against MT cross-reacts with hamster MT-II protein. However, a correlation has been established between cellular MT levels and resistance to metals toxicity (24). Metallothioneins can bind heavy metals such as copper or cadmium and protect cells against their cytotoxicity (15). It has been reported that the overexpression of the transfected human MT-II gene in Chinese hamster cell lines had lead to a 2–6-fold resistance to CdCl2 of these cells (23, 25). Thus, we analyzed cell survival to CdCl2 toxicity to determine whether MT-II mRNA overexpression in V-H4 cells is associated with increased MT-II activity. A comparison of V79 and V-H4 cells demonstrated that V-H4 cells are 2-fold more resistant to CdCl2 toxicity than parental cells, the DL50 values (dose required to reduce cell survival to 50%) of V79 and V-H4 being 0.15 and 0.40 mM, respectively (Fig. 2). These results confirm that V-H4 cells possess higher levels of biologically active MT-II proteins than the parental V79 cells.

Antisense Down-regulation of MT-II Expression—To establish whether MT-II overexpression was directly involved in MMC hypersensitivity of V-H4 mutant cells, we used antisense oligonucleotides to down-regulate MT-II protein expression. Cells were transfected with a 18-base antisense oligonucleotide hybridizing downstream the ATG translational start site of MT-II mRNA or with the corresponding sense sequence. These antisense oligonucleotides were previously used to down-regulate MT-II protein expression in V79 cells (18). Decreased MT-II expression by antisense oligonucleotides was assessed by clonogenic assay following CdCl2 treatment (Fig. 3A). After electrophermeabilization with the antisense MT-II oligonucleotide, V-H4 became as sensitive as V79 cells to CdCl2, because DL50 value was decreased from 0.40 to 0.22 mM. Sense oligonucleotide had no effect on V-H4 survival (Fig. 3A). These results show that antisense MT-II oligonucleotides had efficiently decreased the MT-II protein content in V-H4 cells.

We then investigated the consequences of MT-II down-regulation on MMC resistance of the mutant cells. After electrophermeabilization with oligonucleotides, V-H4 cells were treated for 1 h with increasing doses of MMC as described under “Experimental Procedures.” V-H4 cell survival showed no significant difference with or without treatment with the sense oligonucleotides (Fig. 3B). In contrast, a pre-treatment of V-H4 cells with antisense oligonucleotides significantly increased cell resistance to MMC, because DL50 value increased from 50 to 80 ng/ml (Fig. 3B). These results imply that MT-II overexpression contributes, at least in part, to MMC hypersensitivity of V-H4 mutant cells.

Inhibition of NF-κB Activation in MT-II Overexpressing V-H4 Cells—The role of MT-II in detoxification processes by scavenging toxic molecules is well characterized, and the increase of intracellular levels of metallothionein has usually been associated with the development of resistance to the cytotoxic effects of some alkylating agents (16). However, another potential role of MT-II in the regulation of homeostatic cellular processes following drug treatment has also been reported (23, 25). Recent data have indeed suggested a role of MT-II in the regulation of NF-κB, a “cell survival” transcription factor (26). The Rel/NF-κB family of transcription factors are activated by a wide range of stimuli including DNA damage, cytokines, and free radicals (27). In unstimulated cells, NF-κB is maintained in an inactive state in the cytoplasm by complexing with members of the IκB family such as IκB-α and IκB-β (28). Upon stimulation, IκB-α is rapidly phosphorylated on two serine residues, which target the inhibitor protein for ubiquitination and subsequent degradation by the 26 S proteasome complex (29). NF-κB is then translocated to the nucleus and activates the transcription of a variety of genes including cytokines, cell cycle regulatory proteins, as well as anti-apoptotic proteins (30). MT-II would inhibit the activation of NF-κB and thus prevent transcription of an array of genes implicated in cell survival and control of apoptosis (31). To further characterize the molecular pathway involving MT-II in cellular responses to MMC treatment and how its overexpression can modulate cell survival in V-H4 cells, we have studied NF-κB activation in these cells. The activation of NF-κB was analyzed after MMC treatment by EMSA through its capacity to bind a specific DNA consensus sequence. Specificity of binding was confirmed by competition with an excess of unlabeled oligonucleotide (Fig. 4A, lanes 11 and 12).

Similar low levels of steady-state activation of NF-κB were observed in both resistant and sensitive cell lines (Fig. 4A, lanes 1 and 6). In wild-type V79 cells, there was a clear activation of NF-κB following MMC treatment, which occurred 30
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![Graph]

**FIG. 3.** A, down-regulation of MT-II protein expression by antisense oligonucleotides. V-H4 cells were electroporated with 10 μM oligonucleotides. 24 h after electroporation, cell survival was determined by clonogenic assay 8 days after treatment. Percent cell survival of V-H4 cells pretreated with sense or antisense oligonucleotides is shown as a function of CdCl₂ dose. Data are derived from three independent experiments done in duplicate for each CdCl₂ concentration and are presented as mean values ± S.E. B, MT-II antisense treatment enhances resistance of V-H4 cells to MMC. V-H4 cells were electroporated with 5 μM oligonucleotides. 15 h after electroporation, cell survival was determined by clonogenic assay 8 days after treatment. Percent cell survival of V-H4 cells pretreated with sense or antisense oligonucleotides is shown as a function of MMC dose. Data are derived from three independent experiments done in duplicate for each CdCl₂ concentration and are presented as mean values ± S.E.

![Graph]

**FIG. 4.** A, inhibition of activation of NF-κB by MMC treatment in V-H4 cells. V79 and V-H4 cells were treated for 1 h with 500 ng/ml MMC (lanes 2–5 and 7–10) or left untreated (NT) (lanes 1 and 6). Nuclear extracts were prepared at various times after drug addition, and EMSA was performed with 10 μg of nuclear extracts by using the interleukin-2 consensus NF-κB binding site as probe. The specificity of the binding was confirmed by competition with a 100-fold excess of unlabeled oligonucleotides in cell extracts from V79 and V-H4 cells (lanes 11 and 12, respectively). Relative DNA binding activity was calculated as the ratio of the radioactivity of the NF-κB site binding band in treated cells to that in non-treated cells. B, antisense MT-II protein down-regulation increases NF-κB activation. V-H4 cells were treated for 1 h with 500 ng/ml MMC 15 h after electroporation with the antisense oligonucleotide. Nuclear extracts were prepared 30 min (lane 1) and 90 min (lane 2) after drug addition, and EMSA was performed as presented previously.

min following the addition of a drug and was maximal (~5-fold increase of binding activity compared with untreated cells) after 90 min (Fig. 4A, lanes 2–5). By contrast, only a very weak NF-κB activation was observed in V-H4 mutant cells (~1.4-fold increase of binding activity) (Fig. 4A, lanes 7–10).

We next determined whether MT-II protein overexpression was involved in impaired NF-κB activation after MMC treatment in V-H4 mutant cells. NF-κB activation was analyzed following MMC treatment in mutant cells after electroporation with 5 μM antisense MT-II oligonucleotides (Fig. 4B). Our results showed that MT-II protein down-regulation resulted in a significant increase of NF-κB binding activity following MMC treatment in V-H4 cells (Fig. 4B, lanes 1 and 2). These findings indicate that MT-II overexpression inhibits directly or indirectly NF-κB activation following MMC treatment in V-H4 cells.

To evaluate the specificity of this response, we subsequently examined NF-κB activation following TNFα treatment, which is known to activate this anti-apoptotic transcription factor. In V79 cells, a strong increase of NF-κB binding activity was measured 1 h after TNFα treatment, and this activation persisted up to 24 h (Fig. 5A, lanes 1–5). In V-H4 cells, we observed a significantly delayed NF-κB activation following TNFα treatment (Fig. 5A). Indeed, significant NF-κB activation was only visualized 24 h following TNFα treatment in mutant cells (Fig. 5A, lanes 6–10). To elucidate whether the impaired activation of NF-κB by MMC and TNFα was because of a general incapability to properly activate NF-κB in sensitive cells, we analyzed NF-κB binding activity following camptothecin treatment (Fig. 5B). CPT inhibits topoisomerase I and provokes accumulation of double-strand breaks in DNA upon subsequent interaction of the DNA-topoisomerase I-CPT complex with a replication fork or a transcription machinery and leads to death (32). Our
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results showed a significant and similar increase of NF-κB binding activity in both cell lines by CPT treatment (Fig. 5B), suggesting that NF-κB activation per se is not defective in V-H4 mutant cells.

Altogether our findings suggest that MT-II protein overexpression suppresses NF-κB activation by MMC treatment in V-H4 cells. Furthermore, we observed an altered NF-κB activation in mutant cells following both MMC and TNFα treatments, suggesting that MMC might activate this transcription factor through a common, or partially common, pathway with this cytokine.

DISCUSSION

Increased sensitivity to cross-linking agents such as MMC is a hallmark of the V-H4 hamster cell line (7). We previously reported that an increased induction of apoptosis contributes to its sensitivity toward DNA cross-linking agents (12). To identify gene(s) involved in the MMC hypersensitivity of these cells, we compared mRNA expression of parental V79 and sensitive V-H4 cells. In this report, we showed that V-H4 cells display a 40-fold increase of steady-state expression of MT-II mRNA compared with resistant parental V79 cells. This observed MT-II mRNA up-regulation is correlated with a biologically active MT-II protein overexpression as shown by the increased resistance of V-H4 cells toward cadmium. At present, we do not know why MT-II is overexpressed in these MMC-hypersensitive cells. The principal mechanism of MT-II regulation lies at the level of transcriptional initiation (33). In addition to a large number of positive regulators of MT-II such as Sp1 and AP (21), a novel regulatory factor PZ120 that binds the human MT-II transcription start site and represses human MT-II gene transcription has been recently identified (34). It is conceivable that the level of PZ120 protein or another unidentified repressor of MT-II basal transcription is involved in the overexpression of MT-II that we found in V-H4 cells.

Because of the nucleophilicity of MT, this protein acts as a scavenger of reactive electrophilic molecules and thus protects against antineoplastic drugs (35, 36). Therefore, our finding that MT-II is constitutively overexpressed in MMC-hypersensitive V-H4 cells was not immediately reconcilable with previous data reporting an increased resistance to anticancer drugs resulting from MT-II overexpression (16, 17). Our observation implies that MT-II does not act in this hamster mutant cell line only as a scavenger for alkylating agents but is also associated with the regulation of homeostatic cellular mechanisms. Consistent with this hypothesis and our data, overexpression of the mouse MT gene under the control of a eukaryotic cellular promoter is reflected in decreased resistance to cross-linking agent in wild-type Chinese hamster cells (23). Moreover, the alkylating agent-sensitive MMC-I hamster cells overexpressing the human MT-II-A gene displayed an increased sensitivity to melphalan and MMC (25). Thus, these studies provide evidence for a deleterious effect of MT overexpression on cellular drug resistance in Chinese hamster cell lines. However, in these reports, there was no information whether this sensitivity to cross-linking agents was a result of necrotic or apoptotic cell death and how MT proteins might regulate cellular drug resistance.

To investigate whether MT-II overexpression was directly involved in MMC hypersensitivity of V-H4 cell line, we used antisense oligonucleotides to down-regulate MT-II protein levels. The efficiency of these oligonucleotides was verified by a well established MT functional assay that measures cell sensitivity to heavy metals such as cadmium and was previously used in hamster cell lines (23, 25) and particularly in V79 cells (18). V-H4 cells displayed a 2-fold increase in CdCl2 sensitivity following MT-II protein down-regulation, confirming the significant antisense inhibition of MT-II. Furthermore, under conditions where MT-II is down-regulated, a marked enhancement of the resistance to MMC was observed in the mutant cell line. Thus, we demonstrate here that MT-II overexpression contributes to MMC hypersensitivity in V-H4 cells.

We next assessed whether MT-II overexpression could be directly involved in MMC sensitivity by interfering with mechanism(s) regulating cell survival. It has been reported that MT may regulate NF-κB activation (26). NF-κB is a transcription factor associated with anti-apoptotic effects particularly through the induction of an array of anti-apoptotic genes (31). The impairment of activation of this transcription factor could thus explain the increased induction of apoptosis in V-H4 cells after MMC treatment (37–39). Our results showed that DNA binding activity of NF-κB following MMC treatment is very weak in V-H4 cells as compared with the strong activation observed in V79 parental cells. Consequently, the NF-κB signaling pathway induced by MMC treatment would be expected to be inhibited in V-H4 cells. On the other hand, similar NF-κB activation following CPT treatment occurred in both sensitive and resistant cells, suggesting that basal activation of NF-κB, which allows this transcription factor to translocate into the
nucleus and to regulate its target genes, is not defective in V-H4 cells. Thus, either DNA lesions generated by MMC and CPT activate NF-κB through different pathways because of differences in the type of DNA damage, or because MMC-mediated NF-κB activation does not involve nuclear damage but a cytoplasmic signal. We therefore compared the ability of TNFsα to trigger the NF-κB activation in mutant and parental cells. Our results showed that NF-κB activation after TNFsα treatment is significantly delayed in V-H4 mutant cells. We may therefore hypothesize that MMC and TNFsα activate NF-κB through a common pathway. A number of studies has indeed postulated that some anticancer drugs may mediate cellular responses through activation of cell surface death receptors such as TNF receptors (40–42). The activation of these death receptors leads to cell survival mediated by NF-κB activation through TRAF2 recruitment (43) or triggers apoptosis following FADD recruitment and caspase activation (40). Therefore, blocking NF-κB activation increases cell death, whereas enhanced NF-κB activity protects cells from death. In agreement with this proposed mechanism, impairment of activation of NF-κB by MMC in p53-defective V-H4 cells results in decreased cell survival and enhanced induction of apoptosis triggered by MMC-mediated damages (12).

How can MT-II regulate NF-κB activation? Activation of NF-κB occurs in response to extracellular stimuli or to chemical and physical stresses, allowing a rapid translocation of NF-κB to the nucleus, usually within <15 min (29). Elevated amounts of MT proteins present in the nucleus and/or cytoplasm of cells at the time of the stimulus may block NF-κB activation. Several possibilities could account for MT-II function as a negative regulator of NF-κB. First, it is well known that MT regulates cellular zinc availability (44). An increase of MT-II levels in V-H4 cells may probably lead to Zn depletion by chelation (45), and this depletion has been shown to result in decreased NF-κB activation (46–48) and induction of apoptosis (49, 50). However, Zn depletion is probably to be linked to a general incapacity to activate NF-κB in response to stress, and we detected similar activation of NF-κB following CPT treatment in both V79 and V-H4 cells. On the other hand, a regulation of NF-κB through a physical interaction with MT has also been proposed. MT would interact with the p50 subunit of NF-κB and might be required to stabilize DNA binding of NF-κB after ZnCl₂ treatment (51, 52). However, this assumption would suggest a positive modulation of NF-κB by MT, whereas our results provide evidence that MT-II overexpression leads to specific inhibition of NF-κB by MMC in V-H4 cells. Finally, one could speculate that MT-II is involved in the regulation of a specific signaling pathway that activates NF-κB. We showed that NF-κB activation in response to CPT is different from that triggered by other stimuli, such as MMC and TNFsα. Our findings clearly indicate the existence of different transduction pathways in rodent cells depending on the nature of the signaling molecules. NF-κB could be coupled to distinct upstream signaling pathways through the use of different IκB proteins. Depending on the abundance and the phosphorylation of IκB inhibitors in different cell types, only subpopulations of IκB-NF-κB complexes might be then activated by a specific stimulus. In conclusion, our present findings provide evidence for a significant contribution of MT-II overexpression to MMC hypersensitivity of p53-defective hamster V-H4 cells through the inhibition of NF-κB activation and enhanced apoptotic killing. Further investigations of the role of MT-II and NF-κB will be necessary to understand the mechanism of cell death induced by DNA cross-linking agents. Identifying the mechanisms of MT-II function in inhibition of NF-κB may lead to the design of agents capable of sensitizing p53-defective tumor cells to cytotoxic or DNA damage-induced apoptosis.

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