Journal of Cellular Biochemistry 89:1222–1234 (2003)

Non-Apoptogenic Killing of HeLa Cervical Carcinoma Cells After Short Exposure to the Alkylating Agent *N*-Methyl-*N*′-Nitro-*N*-Nitrosoguanidine (MNNG)

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Abstract We examined the action of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) on HeLa cells and compared it with that of cisplatin (CP). MNNG directly killed a substantial number of cells within 1 hour and resulted in strong DNA-damage as evidenced by Comet measurements. Despite appearance of DNA lesions, p53 protein was not activated. Analysis of HeLa cells treated with MNNG for 1h, 3h and 6h by flow cytometry and by Hoechst staining did not reveal any sub-G_1 cell population and chromatin condensation/fragmentation characteristic for apoptosis, respectively. Also, no biochemical changes typical for apoptosis such as activation of caspase-3 or release of cytochrome C from mitochondria were detected. Inactivation of PARP-1 reduced the direct cytotoxicity exerted by MNNG. Our results showing that despite appearance of severe DNA lesions after short exposure of HeLa cells to MNNG neither activation of p53 response nor induction of apoptosis occurred implicate that generation of strong DNA damage is not sufficient to stabilize p53 protein in HeLa cells. Our data unequivocally show that the conscientious determination of the type of cell death induced by genotoxic agents is necessary. The assessment of the changes based on at least a few independent criteria is required to discriminate between apoptosis and necrosis. Since the alkylating agents generate DNA strand breaks, the recruitment of methods based on determination of DNA cleavage such as DNA ladder or TUNEL assay for evaluation of apoptosis is not adequate. J. Cell. Biochem. 89: 1222–1234, 2003.

Key words: apoptosis; necrosis; inactivation of PARP-1; Comet assay; single cell gel electrophoresis; p53 activation; AIF translocation; cytochrome c; caspase-3 activation; degradation of PARP-1

Apoptosis is not only an essential process during normal embryonic development, but seems to be a widespread phenomenon regulating a dynamic balance between cell proliferation and cell elimination [for reviews, see Tamm et al., 2001; Vermes and Haanen, 1994] to which the homeostatic control of cell numbers is attributed. Apoptosis is responsible for the discrete elimination of superfluous and useless cells and plays a crucial role in the protection of organisms against development of cancer via removal of severely damaged cells. However, if this process is not properly regulated, the transformed or crippled cells survive and can develop malignant tissues by clonal expansion. Cell injury due to a variety of agents, e.g., ionizing radiation, thermic injuries, chemotherapeutic compounds and chemical insults may lead to apoptosis or necrosis. The typical features of a cell dying either by apoptosis or by necrosis are remarkably conserved within distinct death types.

Cells dying by apoptosis show at different stages of the process characteristic changes such as membrane blebbing, outer membrane leaflet inversion and exposure of phosphatidyl serine, cytosolic condensation, cell shrinkage,

Abbreviations used: 7-AAD, 7-amino-actinomycin D; AIF, Apoptosis Inducing Factor; CP, Cisplatin; DAPI, 4,6-diamino-2-phenylidine; DPQ, [3,4.dihydro-5-[4-(1-piperidinyl)butoxy]-1(^2H)-isoquinoline); Hdm-2; human double minute; HMC, Hoffman modulation contrast; HPV, human papilloma virus; KO, knock-out; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; NF-κB, nuclear factor κB; PARP-1, poly(ADP-ribose) polymerase-1; PD, petri dish; PJ-34, [*N*-(6-Oxo-5, 6-dihydro-phenanthridin-2-yl)-*N*, *N*-dimethylacetamide-HCl]; PVDF, polyvinylidene difluoride; VP-16, etoposide; WCL, whole cell lysate; WT, wild-type.

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Received 24 March 2003; Accepted 2 May 2003
DOI 10.1002/jcb.10586

depolarization of mitochondria, activation of the apoptosome, nuclear condensation, chromatin fragmentation, breakdown of nuclear DNA, degradation of proteins, and finally, formation of well-enclosed apoptotic bodies [Fiers et al., 1999; Olson and Kornbluth, 2001; Tamm et al., 2001]. None of these features is observed in cells dying by necrosis, except for annexin staining of phosphatidyl serine residues, but this may be due to internal access because of leakiness of the cell membrane. A rapid loss of the cell membrane integrity during necrosis is an important criterion allowing to distinguish, at least in an early stage of the process, by which way cells die. During apoptosis, membrane integrity persists until a late stage.

The HeLa cell line derived from a malignant human cervical carcinoma harbours wild-type p53 and retinoblastoma tumor suppressor genes [Schwarz et al., 1985; Durst et al., 1987]. They normally control signaling pathways that regulate cell cycle progression and maintain the integrity of the genome [for reviews, Blagosklonny, 2002; Sherr and McCormick, 2002]. The members of the retinoblastoma family control the checkpoint between G_1 and S cell cycle phases by regulation of the activity of E2F transcription factors which promote transcription of a variety of genes required for cell cycle progression [el-Deiry et al., 1993]. Hypophosphorylated Rb family members bind to E2F proteins, thereby forming complexes and repressing their activity. Stepwise phosphorylation of Rb proteins by cyclin-dependent kinases (cdks) disrupts these complexes and released E2F can stimulate transcription of S-phase genes. However, in response to a variety of stress stimuli, the progression of cell cycle can be negatively affected by wild-type p53 protein [Blagosklonny, 2002]. Activated p53 protein acquires transcriptional activity and upregulates a variety of genes including $p21^{waf1}$ [el-Deiry et al., 1993]. $p21^{waf1}$ directly inhibits the activity of cyclin-dependent kinase complexes and arrests cells in G_1 phase, allowing cells to repair the damage or to induce apoptosis [Blagosklonny, 2002; el-Deiry et al., 1993; Sherr and McCormick, 2002]. However, the high-risk human papilloma virus (HPV) E6 and E7 proteins expressed in HeLa cells exert profound effects on both tumor suppressor proteins. E6 protein inactivates p53 by targeting it for accelerated polyubiquitination and degradation in the proteasome [Scheffner et al., 1990]. E7 oncoprotein binds to hypophosphorylated Rb proteins resulting in destabilization and the disruption of the Rb/E2F repressor complexes [Heck et al., 1992; Slebos et al., 1994]. Thus, the E6 and E7 oncoproteins disrupt the cellular response to genotoxic stress stimuli and HeLa cervical carcinoma cells display an aberrant checkpoint control [Slebos et al., 1994].

We recently raised the question of whether apoptosis can be induced in HeLa cells by chemotherapy. We observed that therapy with cisplatin induced apoptosis [Horky et al., 2001] and reactivated wt p53 protein in HeLa cells [Wesierska-Gadek et al., 2002]. The apoptotic process induced with cisplatin or other anticancer drugs (unpublished data) in HeLa cells is a slow, biphasic process executed within 24 h. Surprisingly, it has been previously reported by Kumari et al. [1998], that a short treatment of human HeLa cells with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a strong alkylating agent, upregulated p53 protein and induced apoptosis within a few minutes. The rapid induction of apoptosis by the genotoxic agent was surprising because even the fast apoptotic process initiated by the activation of death receptors takes place within a few hours. Moreover, it has been reported that exposure of cells to MNNG at genotoxic doses for 24 h did not induce apoptosis [Roser et al., 2001].

In the present article, we examined the effect of MNNG on HeLa cells. MNNG damaged the integrity of the cell membrane and killed a substantial number of cells within 3 h. During this period of time, no changes characteristic for apoptosis could be detected. MNNG resulted in a strong DNA damage as evidenced by Comet assay. Despite appearance of severe DNA lesions, p53 protein was not activated. Cleavage of PARP-1 was detected after 6h MNNG action and was not accompanied by activation of caspase-3. We observed that the susceptibility of cells to cytotoxic action of MNNG depends on the PARP-1 status. Pretreatment of HeLa cells with a PARP-1 inhibitor reduced MNNG induced cell membrane disruption and cytotoxicity as evidenced using 7-AAD accumulation and the CellTiterGlo assay, respectively. Moreover, inactivation of PARP-1 rendered mouse cells resistant to direct cytotoxicity exerted by MNNG. The cell membrane integrity persisted during MNNG treatment for 6 h. In response to genotoxic stimuli, cells initiate two different pathways of cell death: apoptosis and necrosis.

The MNNG induced disruption of the cell membrane associated with cell death indicates that HeLa cells die by necrosis.

MATERIALS AND METHODS

Drugs and Chemicals

Cisplatin (PLIVA-Lachema, Brno, Czech Republic), doxorubicin and etoposide (VP-16) (Calbiochem-Novabiochem, La Jolla, CA), MNNG (Sigma Co., St. Louis, MO) and inhibitors of PARP-1 (DPQ and PJ-34) (Alexis Corporation, Lausen, Switzerland) were used in this study. Doxorubicin, etoposide and DPQ were dissolved in DMSO. CP, MNNG and PJ-34 dissolved in culture medium as a stock solution were added to the cells immediately after preparation. A final concentration of DMSO was lower than or equal 0.1%.

Cell Culture

The human cervical carcinoma cell line $HeLaS_3$ (American Type Culture Collection) was cultured in RPMI medium supplemented with 10% foetal calf serum. Cells were grown to 60–70 % confluence and then treated with 5 μM and 50 μM MNNG or with anti-cancer drugs (2 μg/ml doxorubicin, 10 μg/ml VP-16, or 40 μM cisplatin) for indicated periods of time. In some experiments, cells were pretreated with PARP-1 inhibitors: 5 μM DPQ or with 1 μM PJ-34 for 1 h. Normal mouse cells (A-19) and cells lacking PARP-1 (A-11) [Wang et al., 1995] were used in some experiments.

Antibodies

We used the following antibodies: monoclonal anti-p53 antibody DO-1, a kindly gift from Dr. B. Vojtesek (Masaryk Memorial Cancer Institute, Brno), monoclonal anti-PARP-1 antibodies (C-2-10) from Dr. G. Poirier (Laval University, Quebec, Canada). Monoclonal anti-AIF antibody (E-1) and polyclonal anti-NF-κB p65 antibody (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-cytochrome c antibody (clone 7H8.2C12) was from BD PharMingen International (San Diego, CA) and polyclonal anti-caspase-3 was from Dako (Glostrup, Denmark). Monoclonal anti-actin (clone C4) antibodies were from ICN Biochemicals (Aurora, OH). Fluorescein conjugated mouse monoclonal antibodies M30-CytoDEATH recognizing only a caspase cleavage product of cytokeratin 18 were from Roche Molecular Biochemicals (Vienna, Austria). Appropriate secondary antibodies linked to horseradish peroxidase (HRP) or fluorochromes (Cy-2 and Cy-3) were from Amersham International (Little Chalfont, Buckinghamshire, England).

Drug Cytotoxicity In Vitro

Sensitivity of cells to the carcinogen was determined by CellTiterGlo Luminescent Cell Viability Assay (Promega Corporation, Madison, WI) as previously described [Lövborg et al., 2002]. This is a homogenous method for determining the number of viable, metabolically active cells based on quantification of the ATP concentrations. Additionally, dye exclusion test using the vital dye 7-amino-actinomycin D (7-AAD) (BD Biosciences Pharmingen, San Diego, CA) was performed. Cells seeded in Petri dishes were exposed to MNNG for indicated periods of time. Thereafter untreated control and MNNG treated cells were washed twice with PBS and then 5 μl of 7-AAD ready solution (BD Biosciences Pharmingen) was added to 1 ml PBS. After 15 min, the cells were inspected by microscopy. Fluorescence was monitored in the far red range spectrum, and the total cells were visualized by Hoffman modulation contrast (HMC).

Comet Assay

The generation of DNA-strand breaks was assessed by the single cell gel electrophoresis assay performed under alkaline conditions [Singh et al., 1988; Tice et al., 2000]. The experiments were carried out according to the guidelines published by Tice et al. [2000]. Untreated HeLa control and cells exposed for 1 h to 5 μM and 50 μM MNNG were harvested in PBS and the viability of cells was determined using the Trypan blue exclusion test. Only samples exhibiting a viability of at least 80% were used for DNA-migration analysis. Cell suspensions (1×10^5 cells) were mixed with LMP agarose and spread on agarose-precoated slides. After a short incubation at $+4^{\circ}C$ cells were lysed for 1 h and submitted to horizontal electrophoresis at 25 V for 30 min under alkaline conditions. After electrophoresis slides were neutralized and stained with propidium iodide (20 μg/ml). For each experimental point at least three cultures were analyzed. From each culture, 50 cells were evaluated. Comet tail length (μm) and tail moment were measured

under a fluorescence microscope (Nicon Model 027012) using an automated image analysis system based on a public domain NIH image program [Helma and Uhl, 2000].

Detection of Apoptotic Changes in Single Cells

Cells grown on coverslips were treated with MNNG or with CP for indicated period of times, washed three times in PBS and immediately fixed in ice-cold methanol for 20 min and washed in PBS, saturated in 5% BSA in PBS for 1 h and subsequently incubated for 1 h with fluorescein conjugated MAb M30-CytoDEATH antibodies (Roche, Vienna, Austria) recognizing only caspase-3 cleaved cytokeratin 18 and extensively washed in PBS [Wesierska-Gadek et al., 2002]. Thereafter, air-dried preparations were covered with mounting medium containing DAPI and inspected under the fluorescence microscope (inverted microscope Eclipse TE300, Nikon Corporation, Tokyo).

For evaluation of nuclear morphology, cells were fixed in 3.7% paraformaldehyde in cacodylate buffer and stained by Hoechst 33258 dissolved in PBS at a final concentration of 1.5 μg/ml. Preparations were then washed four times and air-dried.

Measurement of DNA of Single Cells by Flow Cytometry

The measurement of DNA content was performed by flow cytometric analysis based on a slightly modified method of Vindelov et al. [1983] as described previously in detail [Wesierska-Gadek and Schmid, 2000]. Propidium iodide stained cells were measured using the Becton Dickinson FACScan.

Fractionation of Cells

In experiments designed to examine the release of distinct proteins from mitochondria during the execution of apoptosis, buffer containing 250 mM sucrose and low concentration of digitonin [0.05% digitonin, 250 mM sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM EDTA, 1.5 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT] was used [Fiskum et al., 2000]. The cells were gently broken by 20–25 strokes with a glass homogenizer. After homogenization, the cell homogenates were transferred to a Falcon tube and nuclei, unbroken cells and large debris were removed by centrifugation at 800*g* for 10 min at +4°C. Supernatants containing mitochondria were transferred into a new tube and were sequentially centrifuged at 22,000*g* for 15 min at +4°C. The resulting supernatants were saved as cytosolic extracts at −80°C until further analysis. The mitochondrial pellets were lysed with buffer containing 1% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1 mM EDTA, and 2 mM DTT.

Electrophoretic Separation of Proteins and Immunoblotting

Total cellular proteins or proteins of the distinct subcellular fractions dissolved in SDS sample buffer were separated on 10% or 15% SDS slab gels and transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF) (Amersham International, Little Chalfont, Buckinghamshire, England). Equal protein loading was confirmed by Ponceau S staining. Blots were incubated with specific primary antibodies and the immune complexes were detected autoradiographically using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescent detection reagent ECL+ (Amersham International, Little Chalfont, Buckinghamshire, England) [Wesierska-Gadek and Schmid, 2000; Wesierska-Gadek et al., 2002]. In some cases, blots were used for sequential incubations.

RESULTS AND DISCUSSION

The maintenance of the integrity of cell membrane is an essential criterion facilitating to distinguish which cellular pathway was activated in response to stress stimuli: apoptosis or necrosis. Whereas during apoptosis the integrity of cell membrane persists until late, its early failure seems to be characteristic for necrosis. Therefore, we examined the direct cytotoxicity of MNNG on HeLa cells by a dye exclusion assay using the vital dyes Trypan blue and 7-AAD. Viable cells with intact membranes exclude dyes, but the membranes of dead and heavily damaged cells are permeable to Trypan blue and 7-AAD. Treatment with 5 μM MNNG for 1 h resulted in an increased accumulation of Trypan blue (about 10–15%) and 7-AAD in HeLa cells as compared to the untreated control. At 50 μM MNNG the proportion of Trypan-blue positive cells increased about twofold. After 3 h at 50 μM MNNG almost all HeLa cells accumulated the dyes, thereby evidencing high cytotoxicity of MNNG. The inspection of

Hoechst stained samples revealed occurrence of a number of mitotic and occasionally apoptotic cells in untreated controls (Fig. 1A). The incidence of apoptosis in untreated controls was very low (under 1%). After 1 h at 50 μM MNNG, no increase of apoptosis was observed. Occasionally (under 1%), apoptotic cells were found, but only when apoptotic cells were also detected in untreated controls. However, alteration of chromatin structure induced after MNNG treatment became evident after 1 h. In the perinucleolar regions, stronger chromatin staining was detectable indicating a DNA damage. Different staining patterns were observed after short- and long-term exposure. After 3 h exposure to MNNG, a large number of cells lost the bottom adherence and became floating. The floating cells were dead as revealed by analysis of the ATP level using CellTiterGlo Luminescent Cell Viability Assay (not shown). Thus, the density of the adherent cell population significantly decreased after 3 h MNNG action (Fig. 1A). Moreover, in a number of nuclei the intensity of the Hoechst staining was reduced implicating loss of dye intercalation due to DNA breakage. A completely different staining

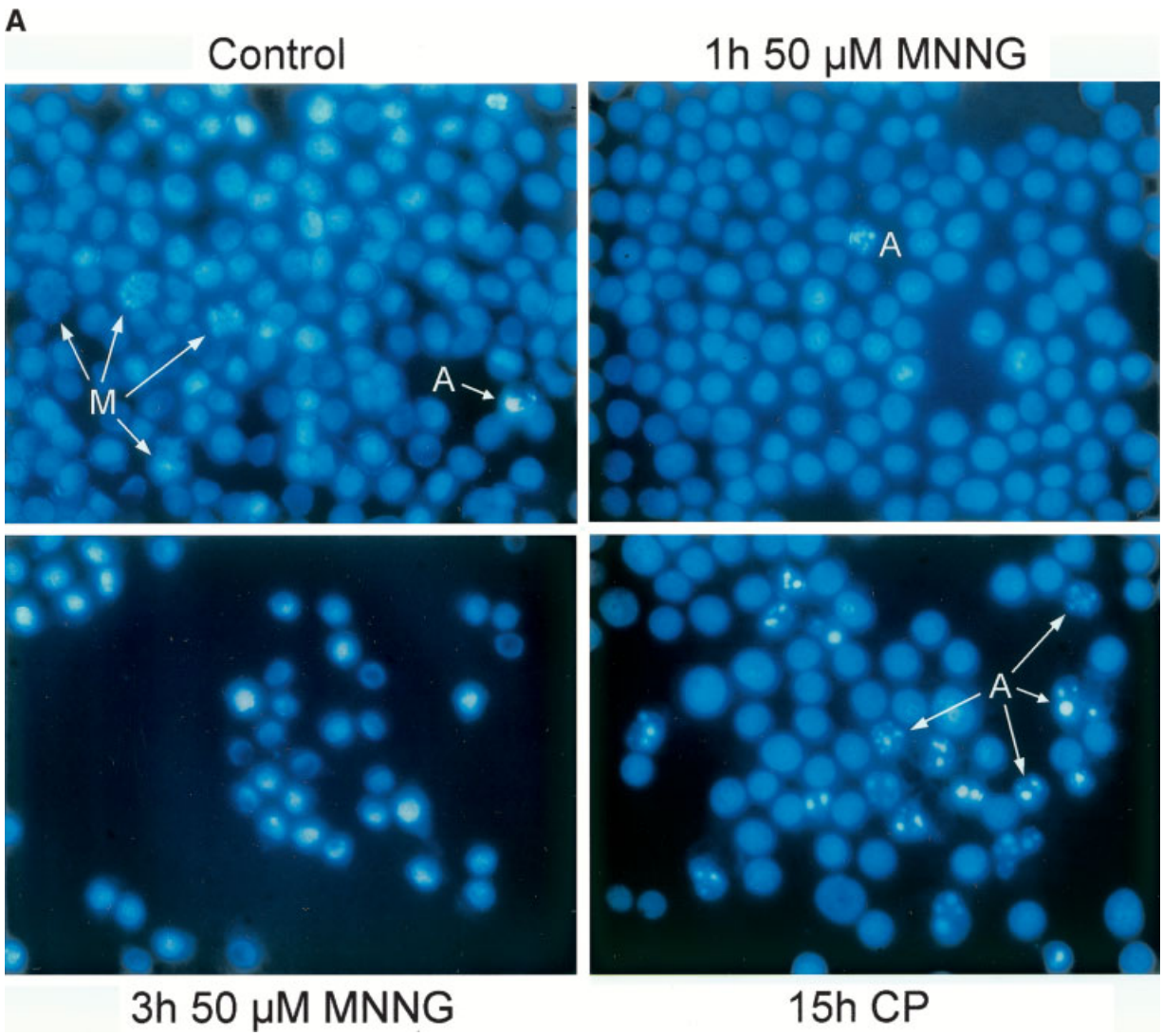

Fig. 1. No induction of apoptosis in HeLa cells by MNNG. **A**: Exponentially growing HeLa cells treated with 50 μM MNNG for 1 and 3 h or with 40 μM CP for 15 h were fixed with 3.7% paraformaldehyde and stained with Hoechst 33258. Mitotic cells (M) and cells in different stages of apoptosis (A) are indicated by arrows. **B**: HeLa cells growing in chambers were treated with 50 μM MNNG for 1 h or with 40 μM CP for 5 h. Then, untreated control and treated cells were methanol-fixed and stained with CytoDEATH30 antibody, recognizing caspase-cleaved cytokeratin 18. Cells were visualized by Hoffman Modulation Contrast (HMC). Nuclei were stained with DAPI dissolved in mounting medium.

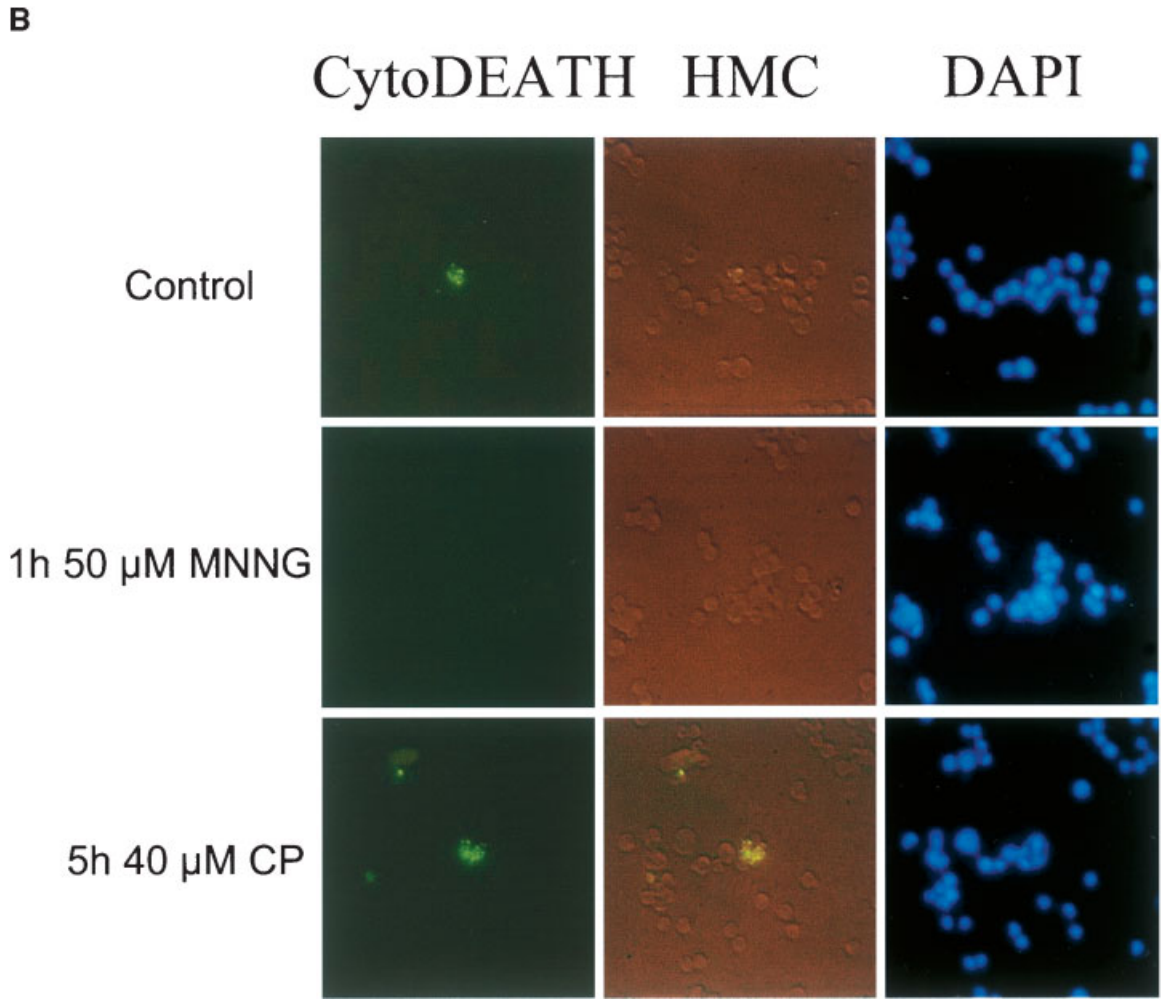

Fig. 1. (*Continued*)

pattern was detected in cells treated with CP. In response to CP HeLa cells underwent apoptosis as evidenced by characteristic condensation of the nuclei and chromatin fragmentation (Fig. 1A). The parallel slides stained with CytoDEATH revealed strong positive signals for caspase cleaved cytokeratin 18 in CP treated HeLa cells (Fig. 1B). However, no CytoDEATH positive staining was detected in MNNG treated cells (Fig. 1B). In the next step, we stained control and MNNG or CP treated cells with propidium iodide and nuclear DNA was measured by FACS analysis. No difference in the profiles obtained from untreated control and cells treated with MNNG for 3 and 6 h was observed (Fig. 2). After exposure of HeLa cells to 50 μM MNNG for 15 h, a small subpopulation (about 10%) of damaged cells (cell debris) or cells with reduced DNA content was detected (Fig. 2). Since after 3 h MNNG treatment, the majority of HeLa cells accumulated vital dyes, this subpopulation of cells seems to represent dead cells. However, after treatment of HeLa cells with CP for 15 h a substantial population of sub G_1 cells (about 50%) appeared. The frequency of hypoploid cells after 15 h CP correlated closely with the frequency of cells exhibiting typical apoptotic fragmentation of chromatin (Fig. 1B) thereby indicating that this is a cohort of apoptotic cells. Thus, the lack of hypoploid HeLa cells representing cells undergoing apoptosis after treatment with MNNG for 3 and 6 h substantiated our previous observations assessed by CytoDEATH and Hoechst staining. Since MNNG is a strong alkylating agent, we determined the DNA damage by the single cell gel electrophoresis method (Comet assay). The cells were exposed to MNNG for 1 h at a

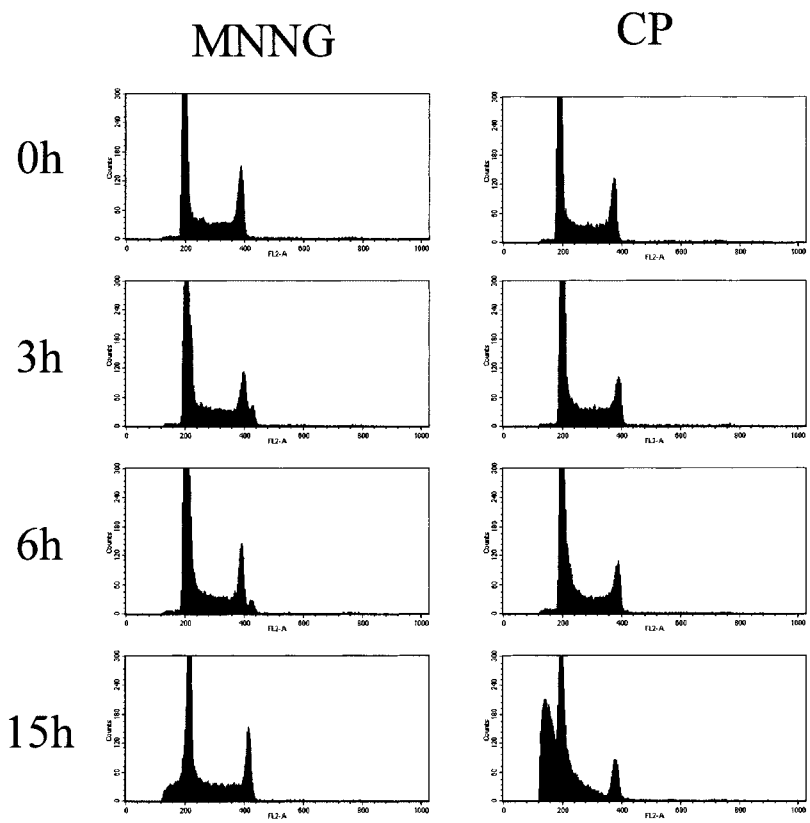

Fig. 2. The effect of MNNG and CP on the distribution of HeLa cells in distinct cell cycle phases determined by flow cytometry. Exponentially growing control HeLa cells and cells treated with 50 μM MNNG or with 40 μM CP for 3, 6, and 15 h were harvested and stained with propidium iodide. Measurement of DNA content was performed on the Becton Dickinson FACScan. Two independent experiments were performed; in each experiment four cultures per time point and treatment were stained and measured. A hypoploid population of cells was detected in cultures treated with CP for 15 h.

final concentration of 5 and 50 μM. MNNG at higher concentration resulted in massive DNA strand breaks. Damaged DNA migrated almost completely out of the nuclei (Fig. 3A) and there remained only residual DNA anchored to the nuclear envelope or in the nucleoli. For these reasons, the Comet assay was not evaluable by the image analysis program in cells exposed to 50 μM MNNG. As depicted in Figure 3A, HeLa cells exposed for 1 h to a tenfold lower concentration of MNNG generated Comets during electrophoresis. The damage of DNA was quantified by determination of tail length (μM) and tail moment according to the international recommendations [Helma and Uhl, 2000; Tice et al., 2000]. As shown in Figure 3B, values of tail length as well as of tail moment after 1 h at 5 μM MNNG increased about fivefold or eightfold, respectively, as compared to untreated controls. Statistical analysis performed with *t*-test revealed the high statistical significance of the increase. These results unequivocally show that MNNG generated in HeLa cells massive DNA-damage even at a low concentration. MNNG, a strong alkylating agent and harmful carcinogen is known to cause methylation in the O^6-position of guanine [Kaina et al., 2001]. The generation of O^6-methylguanine results in mispairing with thymine. Such DNA lesions can be repaired by base excision repair and by mismatch repair pathways [Kaina et al., 2001]. Since the genotoxic shock is known to induce p53 response, we proved the action of MNNG on p53 in HeLa cells. Despite strong DNA-injury, p53 was not induced in HeLa cells after exposure to MNNG (Fig. 4A). However, the anti-cancer drugs such as doxorubicin and CP activated p53 protein in a time dependent mode, whereas etoposide (VP-16) failed to increase p53 in HeLa cells (Fig. 4A). These results are consistent with our previous report that p53 can be reactivated in HeLa cells by CP [Wesierska-Gadek et al., 2002]. The CP induced p53 increase was attributable to its

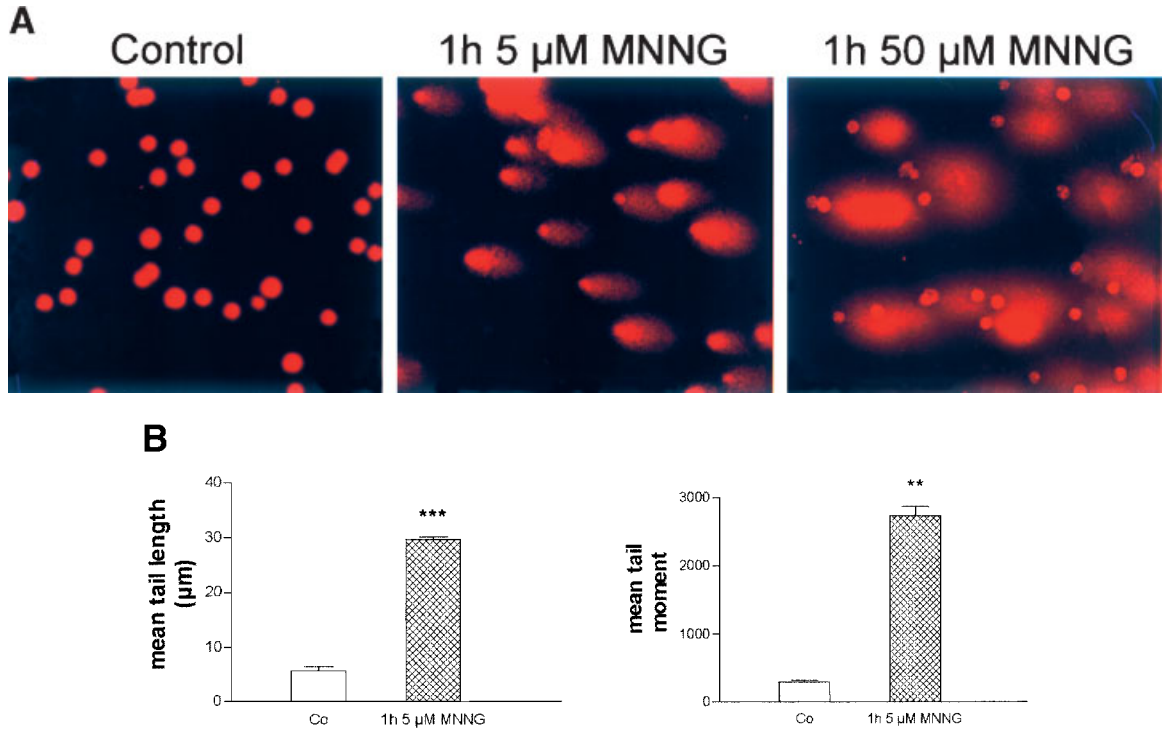

Fig. 3. Strong DNA damage in HeLa cells after treatment with MNNG. Untreated HeLa cells and cells treated with 5 μM or with 50 μM MNNG for 1 h were submitted for single cell gel electrophoresis assay. The DNA was stained with propidium iodide. Three independent experiments were performed; in each experiment cells harvested from three distinct PDs were resolved by electrophoresis. Fifty cells per PD were evaluated. Statistical significance was determined with the *t*-test. Bars indicate means ± SD of 4 PDs per experimental point. Statistical analysis was performed by the unpaired *t*-test. Stars indicate statistical significance valuated with *t*-test: $***P < 0.0001$; $**P = 0.0025$ (a correction for unequal variances was performed with Welch's correction). **A**: Propidium iodide staining. **B**: Values of tail length and of tail moment.

translocation into the nucleolus and to simultaneous repression of HPV encoded E6 oncoprotein [Wesierska-Gadek et al., 2002]. It should be mentioned that Hdm-2 is not the main regulator of p53 stability in HeLa cells. In HPV positive cervical cancer cells a switch from Hdm-2 to E6-mediated degradation of p53 was demonstrated. The virally encoded E6 protein expressed in HeLa cells, which recruits cellular ubiquitin-protein ligase E6-AP activity, targets p53 for accelerated degradation through its enhanced ubiquitination [Scheffner et al., 1990]. Therefore, the reactivation of wt p53 protein in HPV positive cells can be primarily achieved by reduction of the E6 level. Our results are contradictory to the observations of Kumari et al., 1998 who previously reported that p53 was induced in HeLa cells after a 10 min exposure to MNNG. However, this report seems to be questionable due to serious methodological problems and general obstacles. The quality of the p53 detection was poor. The authors did not include positive and negative controls in the blot and did not prove the equal loading of proteins. There is also no information about the antibody used for detection of p53 protein. It is known that p53 protein switches between a latent and an active form and that these two forms differ with respect to reactivity with anti-p53 MAb. Moreover, the increase of p53 concentration in HeLa cells is primarily regulated by modulation of its stability [Scheffner et al., 1990; Wesierska-Gadek et al., 2002]. Therefore, it would be expected that decrease of the E6 oncoprotein level would precede or coincide with upregulation of p53 protein. Despite the importance of this factor for p53 turnover, the authors did not examine it. Considering all these aspects, it seems doubtful that the exposure to MNNG could elongate p53 half-life and result in its translocation into the nucleus within 10 min.

To unequivocally prove whether a cascade of apoptotic events was induced by MNNG within the short period of time, we examined the activation of distinct caspases as well as the

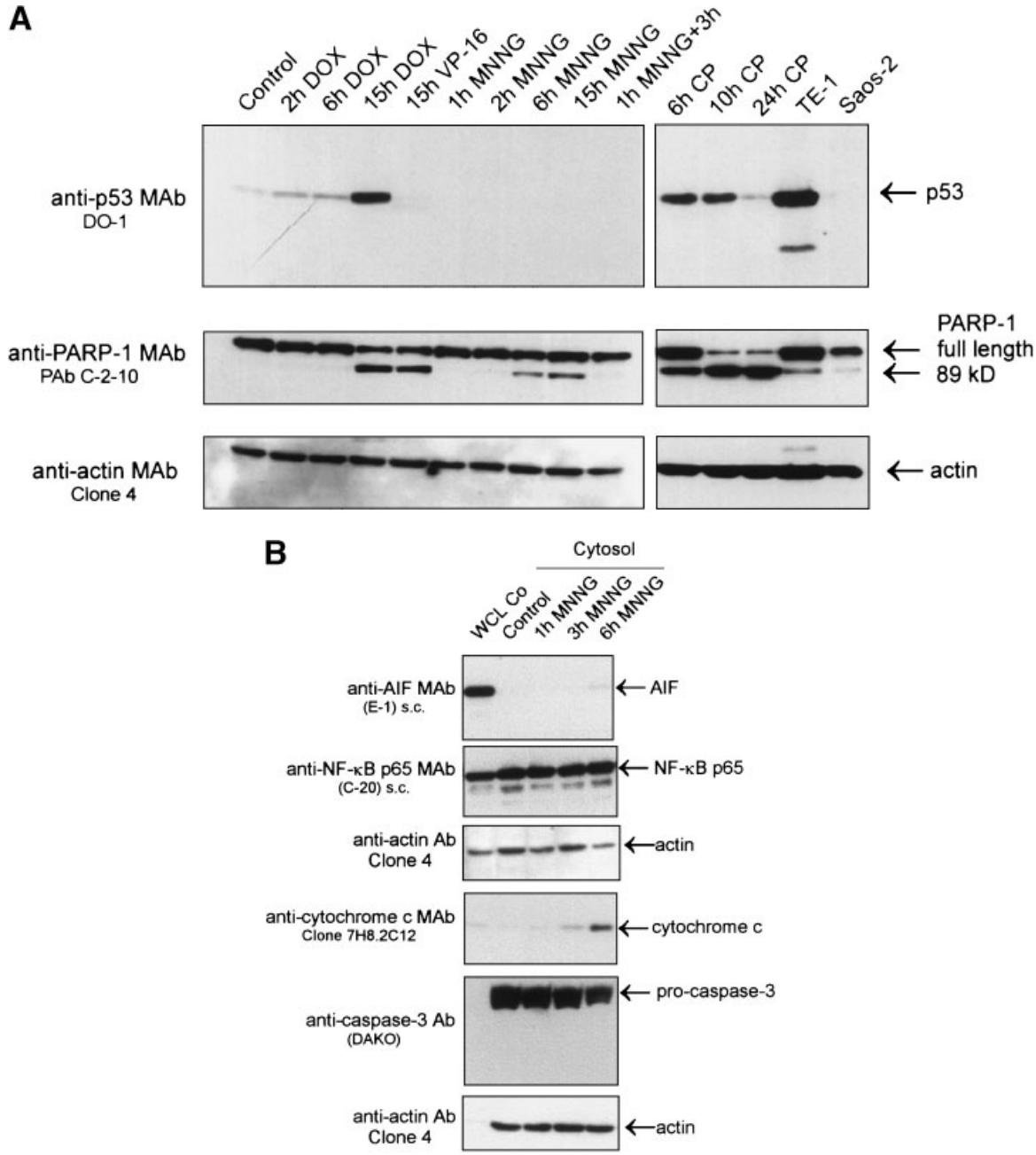

Fig. 4. Lack of p53 induction in HeLa cells after treatment with MNNG. Whole cell lysates or subcellular fractions obtained from HeLa cells treated with 50 μM MNNG or with anti-cancer drugs for indicated periods of time were separated on 10% (**A**) or 10% and 15% (**B**) SDS gels and transferred electrophoretically onto a PVDF membrane. A: The blot was incubated with anti-p53 MAb DO-1. The membrane was sequentially incubated with anti-PARP-1 C-2-10 MAb and then with anti-actin antibodies. Samples prepared from TE-1, a cell line derived from human esophageal cancer exhibiting a mutation at codon 272, and Saos-2 human sarcoma cells were loaded, as a p53-positive and negative control, respectively. BSA (66 kDa) and carbonic anhydrase (30 kDa) were used as molecular weight markers. B: Additional blots were incubated with antibodies against AIF and sequentially with anti-NF-κB antibodies (10%), or with antibodies against cytochrome c and human caspase-3 (15%). In the lower panel protein marker was loaded instead of WCL. The equal protein loading was confirmed by sequential incubation of the blots with anti-actin antibodies.

degradation of nuclear targets by immunoblotting. As expected, poly(ADP-ribose) polymerase-1 (PARP-1), a sensor of DNA damage, was cleaved in HeLa cells exposed to genotoxic agents: an 89 kD carboxyterminal fragment was generated. The extent of PARP-1 degradation differed between treatments. The severe degradation of PARP-1 was observed in HeLa

cells exposed to three anti-cancer drugs. After 10 h CP treatment, PARP-1 was almost completely cleaved. However, in samples exposed to MNNG only negligible amounts of PARP-1 were converted to the 89 kDa truncated fragment and the intensity of full length PARP-1 remained nearly unaffected. The onset of PARP-1 fragmentation was detected after a 6-h exposure of HeLa cells to MNNG. Our results with regard to degradation of PARP-1 and induction of apoptosis differ again from those reported by Kumari et al. [1998]. First, the authors did not present any evidence that MNNG treated HeLa cells underwent apoptotic changes within the observation period. In response to genotoxic stimuli, cells can initiate two different pathways of cell death: necrosis and apoptosis. Therefore, discrimination between both processes based on at least a few independent lines of evidence has to be presented. The conscientious determination of the type of induced cell death is especially important regarding the cleavage of PARP-1. Secondly, the quality of the immunodetection of PARP-1 was poor and it is unlikely that the weak band at 80 kDa occurring only 10 min following onset of MNNG treatment could represent the apoptotic fragment of PARP-1. On the basis of former reports [Horky et al., 2001; Wesierska-Gadek et al., 2002], one could expect that after longer treatment with strong cytotoxic agents a stepwise degradation of PARP-1 should proceed resulting in an increase in the intensity of the 89 kDa fragment accompanied by a decrease of the full length protein. However, the intact protein and 80 kDa fragment were no more detectable after 30 min treatment strengthening the suspicion that the 80 kDa band was caused by unspecific binding. As shown in Figure 4A, the onset of PARP-1 degradation was observed 6 h after exposure of HeLa cells to MNNG and even after 15 h only a negligible portion of PARP-1 was converted to the 89 kDa fragment.

Recently, a direct involvement of PARP-1 in the induction of the apoptotic cascade was described [Yu et al., 2002]. It has been shown that excessive activation of PARP-1 led to exhaustion of the cellular NAD pool. NAD depletion in mitochondria resulted in the release of AIF which activated the following apoptotic cascade. To examine the release of the mitochondrial proteins, we gently fractionated cells by a method described by Fiskum et al. [2000] to avoid any artificial damage of mitochondria during the isolation procedure. The analysis of the cytosolic fraction (Fig. 3B) showed that only low amounts of cytochrome c were released into the cytosol after 6 h MNNG treatment. No translocation of AIF from mitochondria into cytosol (Fig. 3B) during 6-h exposure of cells to MNNG was observed. Only a barely detectable AIF signal was found in the cytosol 6 h post treatment. The sequential incubation of the blot with antibodies against NF-κB, a transcription factor residing in the cytosol in unstimulated cells, revealed comparable NF-κB levels, thereby confirming equal protein loading. The monitoring of caspase-3 activation by immunoblotting of cytosolic samples revealed strong signals for procaspase-3 and no activated p20/p17 form. These results again substantiate our observation that apoptosis was not initiated in HeLa cells within a few hours in response to severe DNA damage generated by MNNG. Our results are consistent with previous observations that MNNG at genotoxic doses did not induce apoptosis in human colon HT 29 cells within 24 h [Roser et al., 2001]. Recently, the effect of MNNG on the expression and intracellular distribution of mismatch repair proteins was described [Christmann and Kaina, 2000]. Agents generating O^6-methylguanidine lesions induced an increase of the nuclear level of MSH2 and MSH6 proteins. The nuclear translocation of mismatch proteins was dose-dependent up to 25 μM MNNG. At higher MNNG doses, the expression of MSH2 protein declined. There is a line of evidence that O^6-methylguanidine and double strand breaks generated by MNNG at low doses trigger the mitochondrial apoptotic pathway [Kaina et al., 2001]. However, the MNNG induced apoptosis seems to be a late event in the cellular defense against the carcinogen, detectable not earlier than 3 days after pulse methylation with MNNG [Ochs and Kaina, 2000] and depends on cell proliferation and DNA replication [Kaina et al., 2001]. The cytotoxic and genotoxic effects exerted by alkylating agents are closely related to the inrinsic DNA repair potential of treated cells. In cells lacking the DNA repair protein O^6-methylguanidine-DNA methyltransferase (MGMT) or expressing it at low levels, generated O^6-methylguanidine is the main lesion. HeLa S3 cells express 620 fmol MGMT/mg protein [Preuss et al., 1996].

In the last series of experiments, we compared the cytotoxic action of MNNG on cells in

which PARP-1 was inactivated. HeLa cells were pretreated for 1 h with DPQ, an inhibitor of PARP-1 (or with PJ-34, not shown) and then exposed to 50 μM MNNG for 3 h. In a parallel experiment the action of MNNG was examined on normal mouse cells and cells obtained from PARP-1 knock-out mice. We determined the integrity of the cell membrane by the accumulation of 7-AAD and the viability of cells by the CellTiterGlo assay. Pretreatment of HeLa cells with inhibitors of PARP-1 significantly reduced the cytotoxic effect of MNNG (Fig. 5A). The proportion of damaged cells accumulating 7-AAD was reduced by about 50% after combined treatment (not shown). On the other hand, the number of viable HeLa cells increased, as

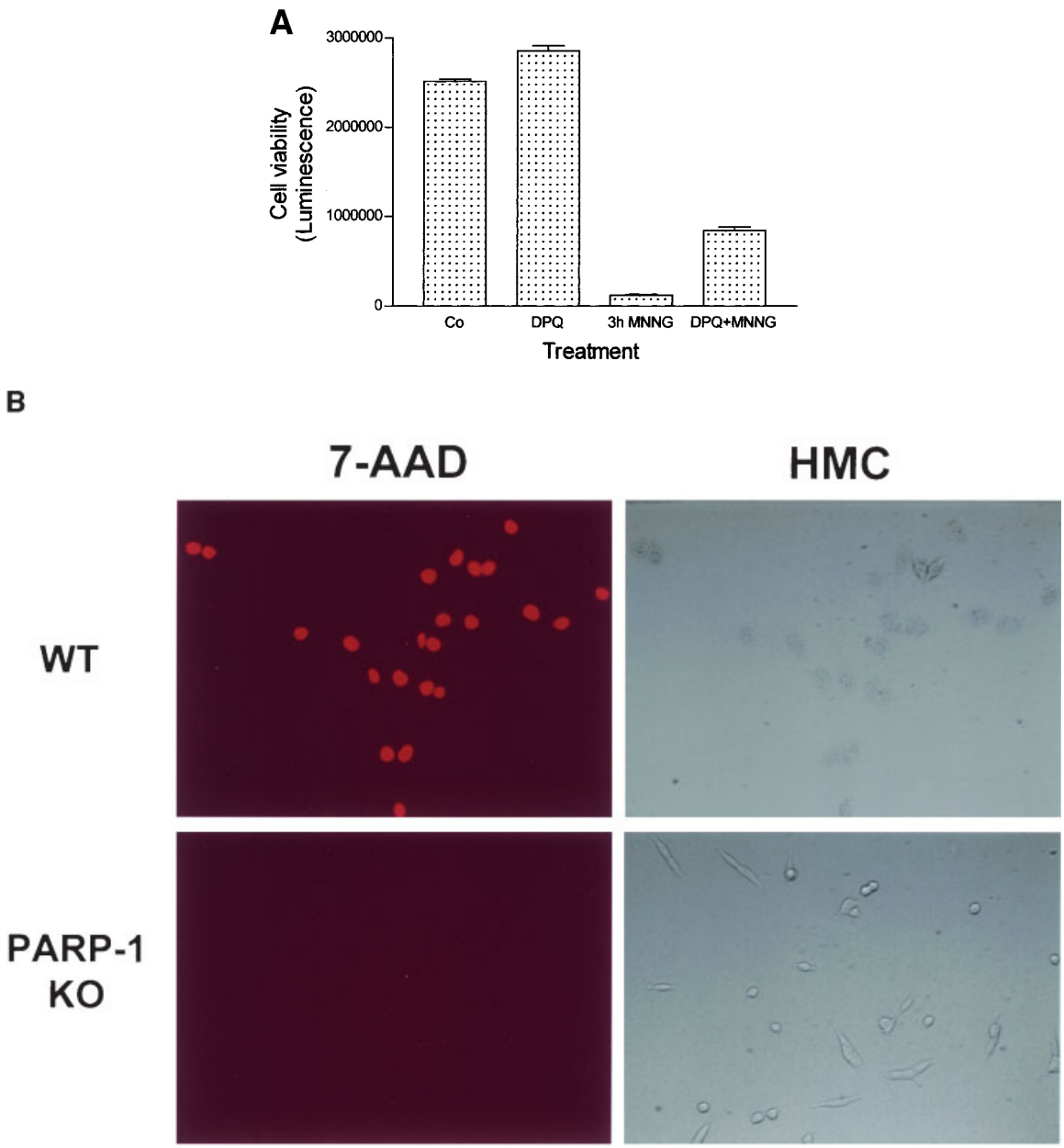

Fig. 5. Inactivation of PARP-1 reduces direct cytotoxicity exerted by MNNG. **A**: HeLa cells were pretreated with 5 μM DPQ for 1 h and then MNNG was added for 3 h to a final concentration of 50 μM. Cell viability was determined by CellTiterGlo and by accumulation of 7-AAD (not shown). **B**: Normal (WT) and PARP-1 knock-out (KO) mouse embryo fibroblasts were treated for 6 h with 50 μM MNNG. Cell viability was determined by CellTiterGlo (not shown) and by accumulation of 7-AAD. All cells on the slide were visualized by Hoffman Modulation Contrast (HMC).

evidenced by CellTiterGlo assay (Fig. 5A), when PARP-1 was inhibited during action of MNNG. Furthermore, we observed that susceptibility of the murine fibroblasts to the cytotoxic action of MNNG depended on their PARP-1 status. Whereas nearly all wild-type fibroblasts died after 6 h treatment with 50 μM MNNG, as evidenced by intracellular accumulation of 7-AAD, PARP-1 knock-out cells remained almost unaffected (Fig. 5B). Thus, our results show that MNNG is highly cytotoxic and generates not only a strong DNA-damage but also impairs the integrity of the cell membrane. The leakiness of the plasma membrane is a characteristic feature of necrosis. MNNG at high concentration generates massive DNA strand breaks that result in dramatic activation of PARP-1 followed by formation of poly(ADP-ribose) chains associated with fast consumption of NAD. This leads to energy depletion and to rapid cell suicide as suggested previously by Berger [1985]. The inactivation of PARP-1 may prevent the damage of the cell membrane and seems to reduce the necrotic cell death following excessive DNA damage. This assumption is in concordance with previous suggestions of Berger [1985].

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