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Strong Induction of p73 Protein In Vivo Coincides With the Onset of Apoptosis in Rat Liver After Treatment With the Hepatocarcinogen *N*-Nitrosomorpholine (NNM)

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Abstract Treatment of rats with genotoxic hepatocarcinogens such as *N*-nitrosomorpholine (NNM) causes severe hepatotoxicity associated with apoptosis of hepatocytes beginning after 12 h. Previously, we reported that after a single administration of high NNM dose p53 protein level increased in liver but not in testis and that the first wave of apoptosis preceded the induction of p53 indicating that apoptosis in liver was driven by a p53-independent pathway. We now show a pronounced upregulation of p73 protein, a p53-related gene product. The increase of p73 α and β occurred already 6 h after NNM administration and preceded the onset of apoptosis by 6 h. Very strong p73 signals appeared 20 and 40 h post-treatment and persisted for a few days, whereas p53 was induced only transiently at 20 and 40 h post-treatment. Immunohistochemical analysis revealed that unlike p53, p73 was detected in the nuclei of hepatocytes undergoing apoptosis. Following the upregulation of p73 levels, the products of several genes regulating DNA repair, e.g., GADD-45 and p53R2 and mediating apoptosis such as apoptosis inducing factor (AIF) were rapidly induced, whereas transient elevation of MDM-2 protein was delayed and coincided temporary with activation of p53 protein. Interestingly, NF-κB another transcription factor responding to cellular stress was activated at 20 h after NNM administration and reached a maximum after an additional 20 h. Our data indicate that activated p73 protein may positively affect the induction and execution of apoptosis in response to genotoxic action of NNM. J. Cell. Biochem. 90: 837–855, 2003.

Key words: TUNEL assay; activation of NF-κB; p53 response; PARP-1 cleavage; caspase activation; cytochrome C release

N-nitroso compounds are highly cytotoxic and have been recognized as potent carcinogens [Moore et al., 1982; Enzmann and Bannasch, 1987; Weber and Bannasch, 1994]. They generate different types of DNA damage including DNA strand breaks [Brambilla et al., 1987]. Acute cytotoxicity of the nitrosamine *N*-nitrosomorpholine (NNM) seems to be stronger than that of dialkyl compounds [Stewart et al., 1975]. The genotoxic risks exerted by these agents depend not only on their cytotoxic potential but are closely related to the capability of the affected cells or organisms to induce a defence response allowing to repair the DNA lesions or to eliminate the severely damaged cells by apoptosis. The proper regulation of the defence strategy reduces the risks of neoplastic transformation [Thorgeirsson et al., 1998].

Insufficient information is available on molecular events preceding the induction and execution of cell death in the liver by genotoxic

Abbreviations used: AIF, apoptosis inducing factor; EMSA, electrophoretic mobility shift assay; GADD-45, growth arrest and DNA damage-45; HRP, horseradish peroxidase; MDM-2, mouse double minute; MEF, mouse embryo fibroblast; NF-κB, nuclear factor κB; NNM, *N*-nitrosomorpholine; PARP-1, poly(ADP-ribose)polymerase-1; PBS, phosphate-buffered saline; p53R2, p53-dependent ribonucleotide reductase; PCNA, proliferating-cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; PNS, post-nuclear supernatant; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; TdT, terminal deoxydinucleotidyl transferase; ts, temperature-sensitive; TUNEL, TdT-dUTP-nick-end-labeling; WCL, whole cell lysate; wt, wild-type.

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hepatocarcinogens. There are contradictory reports on the role of p53 tumor suppressor protein in the management of cellular response in the liver to various hepatocarcinogens. It became clear that there is a tissue variability in the induction of p53-controlled cellular responses. Moreover, there is also variability even in the same tissue context when evaluating the roles of p53 in liver in vivo or in primary hepatocytes. It is well known that metabolic activation of some carcinogens such as *N*-nitrosamines is required for the generation of electrophilic species that can elicit genotoxic effects [Manson et al., 1978].

In normal rat liver in vivo, the oral exposure to nitrosamine NNM triggered apoptosis which was apparently unrelated to p53 [Wesierska-Gadek et al., 1999]. The induction of apoptosis preceded the activation of p53 protein and showed a different spatial distribution than the upregulated p53 protein [Wesierska-Gadek et al., 1999]. Surprisingly, despite a strong tissue and DNA damage, p53 was only moderately induced. It remained unresolved by which mechanism DNA and cell damage resulted in apoptosis.

In recent years, p53 has been found to be a member of a larger gene family. New p53-related genes such as *p73* [Jost et al., 1997; Kaghad et al., 1997] and *p63* [Osada et al., 1998] have been identified. A new *p73* gene encoding a protein with a striking structural and functional homology to p53 is expressed in a full-length form, α, and a few shorter splicing variants [De Laurenzi et al., 1998]. The expression pattern of the p73 splicing variants differs between cell types and may vary between differentiation stages even within the same cell lineage [De Laurenzi et al., 1998].

The p53 tumor suppressor protein is an essential component of one of the pathways activated in response to DNA damage [reviewed by Harris, 1996; Ko and Prives, 1996]. p53 induction results in the inhibition of cell cycle progression or initiation of apoptosis. To exert most if not all of its biological functions, wild-type p53 acts as transcriptional activator on numerous responsive genes that contain specific p53 consensus sequences on their promoter [el-Deiry et al., 1992], such as the GADD-45 [Kastan et al., 1992], MDM-2 [Momand et al., 1992], PCNA [Jackson et al., 1994, Morris et al., 1996], and $p21^{Waf1/Cip1/Sdi1}$ [el-Deiry et al., 1993] or as transcriptional repressor on genes that lack such sequences probably by sequestering transcription factors. Similar to p53, p73 protein can activate transcription of $p21^{Waf1/Cip1/Sdi1}$, a gene involved in cell cycle arrest at G_1 via inhibition of the cyclin-dependent protein kinases 2/4/6 (cdk2) [Fontemaggi et al., 2002] and can induce apoptosis when overexpressed [reviewed by Ikawa et al., 1999; Kaelin, 1999; Levrero et al., 1999]. Earlier studies have shown that unlike p53, p73 was not induced by DNA damage [Kaghad et al., 1997]. However, more recent reports showed that p73 is activated in cultivated cells in response to cisplatin and γ-radiation [Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999] suggesting that p73 may exert distinct functions in the cellular response to different stimuli. The upregulation of p73 protein was tightly coupled with cisplatin or radiation induced p53-independent apoptosis [Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999]. These new data evidencing the role of p73 protein in the initiation of apoptosis were obtained with cultivated cells. To what extent they apply to the situation in the intact organism has so far not been studied. Therefore, since our findings indicate that NNM-induced apoptosis in rat liver was p53-independent [Wesierska-Gadek et al., 1999], the question appeared whether of p73 protein may serve as a trigger for apoptosis after genotoxic injury in the liver. The present work shows a strong induction of p73 expression in rat liver after a single application of the hepatocarcinogen NNM. The p73 increase preceded the onset of apoptosis. Several hepatocyte nuclei in the early stage of apoptosis expressed p73 protein as evidenced by immunostaining. These results strongly suggest that p73 can serve as inducer of apoptosis in the liver following genotoxic injury.

MATERIALS AND METHODS

Plasmids

Plasmids encoding human p73 splicing variants were kindly supplied by Dr. G. Melino (University of Rome Tor Vergata, Rome).

Animals and Treatment

Two independent experiments were performed. Three- to five-week-old male SPF Wistar rats were obtained from the Forschungsinstitut für Versuchstierzucht und Versuchstierhaltung (Himberg, Austria). NNM was dissolved in PBS. Rats (three animals per

time-point) were given a single dose of 250 mg NNM/kg body weight by oral gavage between 8 p.m. and 9 p.m. when the wave of hepatic DNA synthesis was at its peak, as described previously in detail [Wesierska-Gadek et al., 1999]. Controls were treated with solvent (10 ml/kg). In the first experiment, the rats were killed under carbon dioxide anoxia by decapitation and exsanguinated 6, 12, 20, 40, 60, 84, and 156 hours after NNM administration. In the second experimental, series shorter time intervals after NNM administration were additionally included.

Cells and Treatment

Human breast carcinoma MCF-7 cells, human osteosarcoma Saos-2 cells and the human Ewing tumor cell line overexpressing a temperature-sensitive $p53^{val138}$ mutant [Kovar et al., 2000] were used. MCF-7 and Saos-2 cells were treated with 40 μM cisplatin for indicated periods of time. Ewing tumor cell line cultivated at basal temperature (37°C) was shifted-down to 32°C. At 37°C human $p53^{val138}$ protein behaves as oncoprotein. Upon temperature down-shift to 32°C overexpressed $p53^{val138}$ mutant adopted wild-type conformation resulting in the induction of distinct target genes and of apoptosis. At early stages of apoptosis, PARP-1 was cleaved into the 89 kDa fragment by activated caspase-3 [Kovar et al., 2000].

Antibodies

Different anti-p73 antibodies were used in this study. Polyclonal anti-p73 antibodies S460 recognizing α and β isomers were used from AbCam Ltd. (Cambridge, UK), two antibodies reacting solely with the α form (C-17 and ER-13) were from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, CA) and NeoMarkers (Fremont, CA), respectively. Polyclonal antibodies recognizing all p73 isoforms (H-79), antibodies against granzyme B (N-19), antibodies against caspases-9 (H-83) and caspases-3 (L-18), antibodies against p53R2 (C-18), a mouse monoclonal antibody against AIF (E-1) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-p53 antibodies recognizing an epitope in the carboxy-terminus of both wild-type and mutant p53 (PAb421), antibodies reactive with an epitope in the core domain of p53 protein (PAb240), a mouse monoclonal anti-PARP-1 (C-2-10) antibody, a mouse monoclonal anti-MDM-2 antibody (SMP-14), a mouse monoclonal antibody against MCM7 protein (DCS141.2), a mouse monoclonal anti-PCNA antibody (PC-10) and polyclonal anti-waf 1 (Ab-5) antibodies were obtained from Oncogene Research Products (Cambridge, MA). Polyclonal anti-IκB-α antibodies and antibodies recognizing phosphorylated form of p53 at serine 392 were from New England BioLabs, Inc. (Beverly, MA). For Ki-67 polyclonal antibodies from DAKO A/S (Glostrup, Denmark) were used. Anti-GADD-45 (4T-27), anti-NF-κB p50 (C-19), and anti-NF-κB p65 (C-20) were obtained from Santa Cruz Biotechnology, Inc. Polyclonal anti-caspase-3 antibodies from Upstate Biotechnology (Lake Placid, NY) were additionally used. A mouse monoclonal anti-cytochrome C antibody (7H8.2C12) was obtained from BD PharMingen (San Diego, CA). Monoclonal anti-actin antibody was from ICN Biomedicals, Inc. (Aurora, OH). Appropriate secondary antibodies linked to HRP were from Pierce (Rockford, IL).

Histology

Tissue slices of about 3 mm thickness were fixed in 4% buffered formalin and embedded in paraffin. Sections (3–4 μm thick) were stained with anti-p73 antibodies and then counterstained with hematoxylin & eosin (H&E) for evaluation of the hepatocyte morphology. Immune complexes were detected after incubation with secondary antibodies coupled to HRP using 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2 as a substrate as described previously [Wesierska-Gadek et al., 1999].

Determination of Apoptosis in Individual Cells

To assess DNA fragmentation in individual cells we performed the TUNEL enzymatic labeling assay in situ. This widely used method is based on the enzymatic labeling of DNA strand breaks (free 3′-OH termini) with modified nucleotides (e.g., fluorescein-12–dUTP) using exogenous terminal deoxynucleotidyl transferase. Tissue paraffin sections (3–4 μm) were dewaxed and rehydrated and then processed for labeling using the DeadEnd Fluorometric TUNEL Kit (Promega, Madison, WI) according to the procedure recommended by manufacturer's. Samples were counterstained with propidium iodide at a final concentration of

1 μg/ml and immediately evaluated. The number of hepatocytes undergoing apoptosis was determined by TUNEL assay combined with propidium iodide staining. The evaluation of the nuclear morphology allowed to discriminate between hepatocytes undergoing apoptosis and necrosis. At least 4,000 hepatocytes were scored from each sample.

Isolation of Nuclei and Cytosol Fraction From Post-Nuclear Supernatant

To avoid proteolytic degradation of proteins, all isolation steps were performed at +4°C in the presence of the protease inhibitors PMSF and Pefabloc at a final concentrations of 1 mM and 100 μM, respectively. Nuclei were isolated by the method of Tata [1974]. Post-nuclear supernatant was centrifuged at 100,000*g* at 4°C for 1 h yielding a pellet containing mitochondria and a supernatant designated as S-100 [Fiskum et al., 2000].

Determination of Protein Content

Aliquots of isolated nuclei were dissolved in non-reduced SDS-sample buffer. Protein concentration in isolated nuclei, cell lysates and cytosol samples was determined by the DC assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard.

In Vitro Transctiption/Translation Assay

Plasmids encoding distinct p73 splicing isomers were used as templates for the translation assays employing the TnTQuick coupled transcription/translation system (Promega Corporation, Madison, WI). The assay was performed according to the manufacturer's protocol using ^{35}S-labeled methionine (NEN Life Science Products, Boston, MA). Plasmids (2 μg DNA) containing T7 RNA polymerase promoter were added to an aliquot of the TnT Quick Master Mix and incubated in a 50 μl reaction volume for 90 min at 30°C. The synthesized proteins then were analyzed by gel electrophoresis. The radioactively labeled recombinant proteins were detected by autoradiography.

Determination of Caspase-3/7 Activity

The activity of both caspases was determined using the APO-ONE Homogenous Caspase-3/7 Assay (Promega, Madison, WI). This test uses the caspase-3/7 substrate rhodamine 110, bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (Z-DEVD-R100). This compound exists as a pro-fluorescent substrate prior to the assay. Upon cleavage and removal of the DEVD peptide by caspase-3/7 activity and excitation at 499 nm, the rhodamine 110 leaving group becomes intensely fluorescent. Equal amounts of cytosol proteins (S-100) prepared from control and NNM-treated rats were diluted with sucrose buffer to a final volume of 50 μl. Then an equal volume of caspase substrate was added and samples were incubated at 37°C for 2 h. The fluorescence was measured at 485 nm. Sucrose buffer was used as a blank. "No-cell background" values were about 109–315 cpm.

Immunoprecipitation

Nuclear extracts prepared in RIPA buffer (100–300 μg) or aliquots of p73 isomers radioactively labeled during in vitro translation were diluted in PBS and incubated with corresponding antibodies for 1 h at room temperature. Immunoprecipitation was performed as described previously in detail [Wesierska-Gadek et al., 1998]. Prewashed GammaBind-G-Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were used for isolation of immune complexes. Eluted proteins were separated on SDS gels and radioactively labeled proteins were detected by autoradiography using MR film (Eastman Kodak Co., Rochester, NY).

Immunoblotting

Proteins dissolved in reduced SDS sample buffer were loaded on SDS polyacrylamide slab gels, electrophoretically separated and transferred onto polyvinylidene difluoride membrane (PVDF) (Amersham International, Little Chalfont, England, UK). Equal loading of proteins was confirmed by Ponceau S staining. Immunodetection of antigens was performed with specific primary antibodies. The immune complexes were detected autoradiographically using appropriate HRP-linked secondary antibodies and enhanced chemiluminescence detection reagent ECL+ (Amersham International) and LS film (Eastman Kodak Co., Rochester, NY).

Statistical Analysis

Statistical analysis was performed by the ANOVA test followed by Dunnett's Multiple-Comparison test (all treatment groups vs. control).

RESULTS

Evaluation of Apoptosis

Rats sacrificed within the first days after administration of NNM displayed considerable loss of liver tissue. To determine NNM-induced fragmentation of DNA in rat liver, the TUNEL assay was performed. Since after NNM administration not only apoptosis but also necrosis is induced, the discrimination between both processes was necessary. The labeling of free 3′-DNA ends with fluoresceine combined with propidium iodide allowed to evaluate the morphology of positive hepatocytes and fulfilled these criteria. In tissue samples obtained from liver of control animals a weak positive TUNEL reaction was observed only occasionally. Most hepatocytes remained TUNEL negative (Fig. 1A). Six hours after NNM administration hepatocytes in early stages of apoptosis were detected by TUNEL assay. The apoptosis rate was low (about 1.0%) (Fig. 1B). At 12 h, the frequency of apoptosis increased and reached approximately 5% at 20 h. As illustrated in Figure 1A, the overlapping of two fluorochromes: red representing condensed chromatin and green representing sites at which fragmented DNA was fluorescein labeled, in hepatocytes in different stages of apoptosis generated yellow staining. To visualize the global distribution of TUNEL positive staining at 40 h post-treatment, an image at low magnification was shown. At this time-point, the frequency of necrotic hepatocytes significantly increased.

Cleavage of PARP-1 as an Early Marker of Cell Death

Our previous results [Wesierska-Gadek et al., 1999] showed striking correlation between an onset of PARP-1 cleavage and induction of apoptosis after NNM treatment in rat liver. Monitoring of PARP-1 status in the present experiment reproduced and extended former results. First, the formation of proteolytic fragments of PARP-1 in liver nuclei was detected already 6 h after NNM administration (Fig. 2A). At this time-point, the rate of apoptosis as evaluated on basis of morphological features or DNA fragmentation only slightly increased. Furthermore, in contrast to a series of heterogeneous fragments of human PARP-1 appearing after limited proteolysis [Wesierska-Gadek et al., 1999], in the liver of NNM-treated rats, two distinct degradation products lacking the N-terminal part were generated: one at 64 kDa and, in some samples an additional one at 54 kDa (Fig. 2A). In testis, which is not a target of NNM toxicity, immunoblotting revealed only a single band at 116 kDa [Wesierska-Gadek et al., 1999], confirming that cleavage of PARP-1 did not occur in the negative control (not shown).

A comparison of the intensity of full-length protein signal visualized by the monoclonal anti-PARP antibody C-2-10 (after short exposure of blots) (Fig. 2A, lower panel) in the course of time after NNM administration showed a marked decrease of intact PARP-1 beginning at 6 h. Densitometric quantification revealed a reduction of the intensity of the band representing intact PARP-1 by up to 40% in nuclei of NNM-treated rats. Diminution of the level of full-length PARP-1 was accompanied by the appearance of its truncated fragments at 64 kDa and occasionally at 54 kDa beginning at 6 h post-treatment. The size of the larger fragment was consistent with that of the polypeptide generated by granzyme B [Froelich et al., 1996] implicating that the observed cleavage pattern could be attributable to the action of granzyme B. Examination of the granzyme B distribution revealed its accumulation in the isolated nuclei beginning 12 h after NNM administration (Fig. 2B). This observation confirms our assumption that PARP-1 was proteolytically processed by activated granzyme B.

Interestingly, at 40 h post-treatment instead of the 64 kDa COOH-terminal polypeptide a canonical 89 kDa fragment of PARP-1 was generated resembling that produced by caspase-3. The 89-kDa fragment was seen in the human Ewing tumor cell line [Kovar et al., 2000] used as positive control (Fig. 2A). In our 1st experiment, only two rats exhibited this cleavage pattern at 40 h, whereas a 3rd animal showed the 64 kDa fragment. In conclusion, the onset of PARP degradation at 6 h after NNM application accompanies or even precedes the first wave of apoptosis. Moreover, it became evident that two distinct degradation pathways of PARP-1 can be independently induced in vivo in response to the same insult.

Activation of Distinct Caspases After NNM Administration

The generation of a 89-kDa fragment of PARP-1 appearing at 20 h and more pronounced

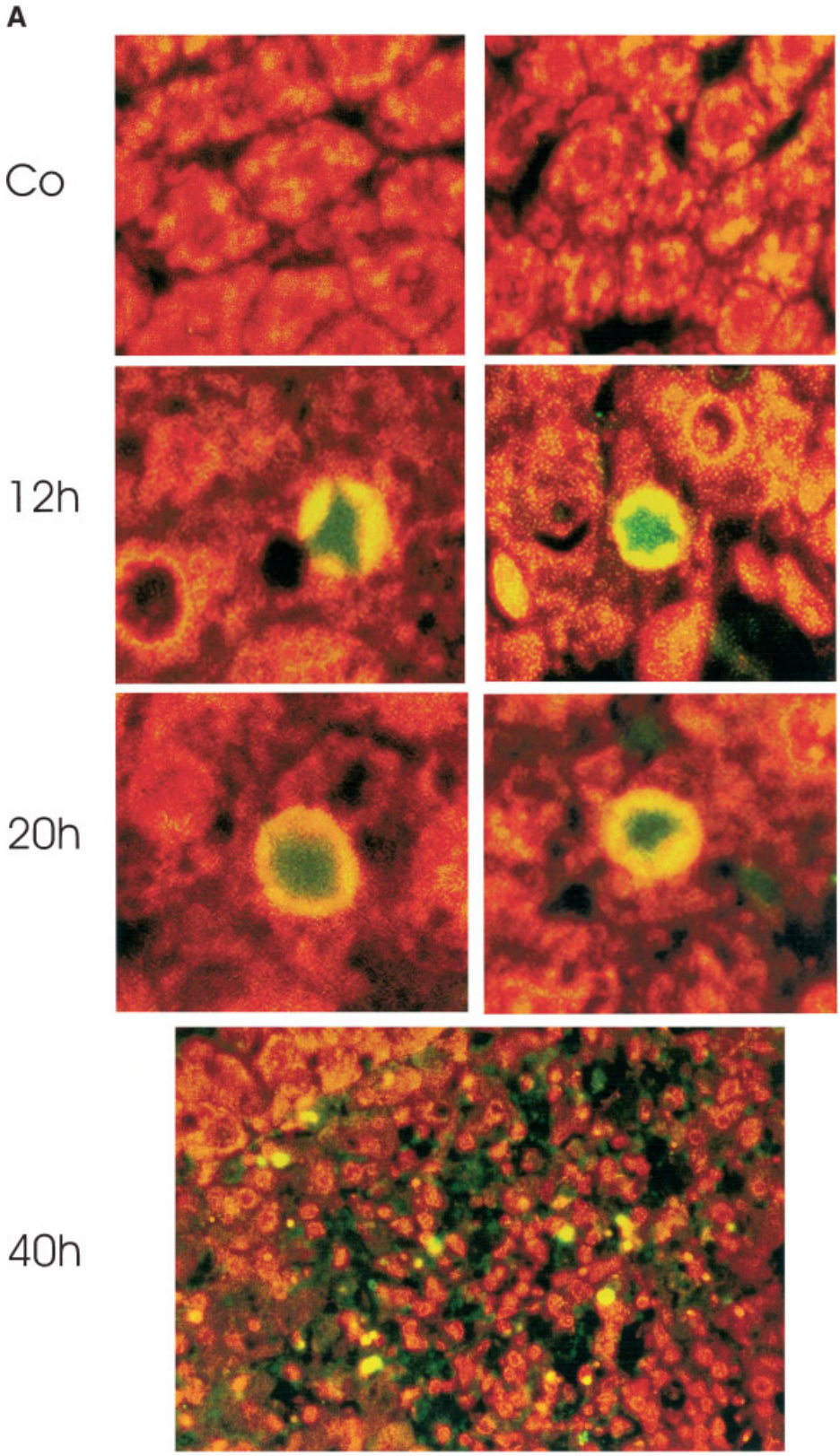


Fig. 1.

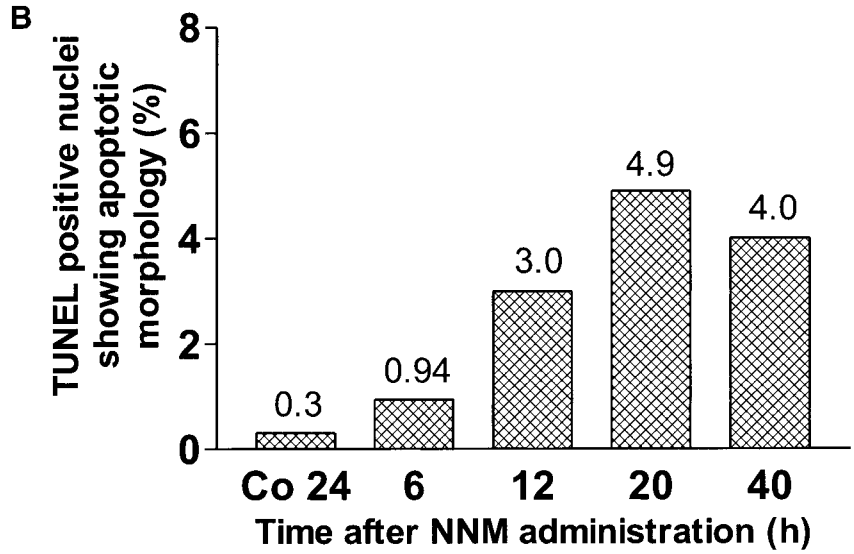


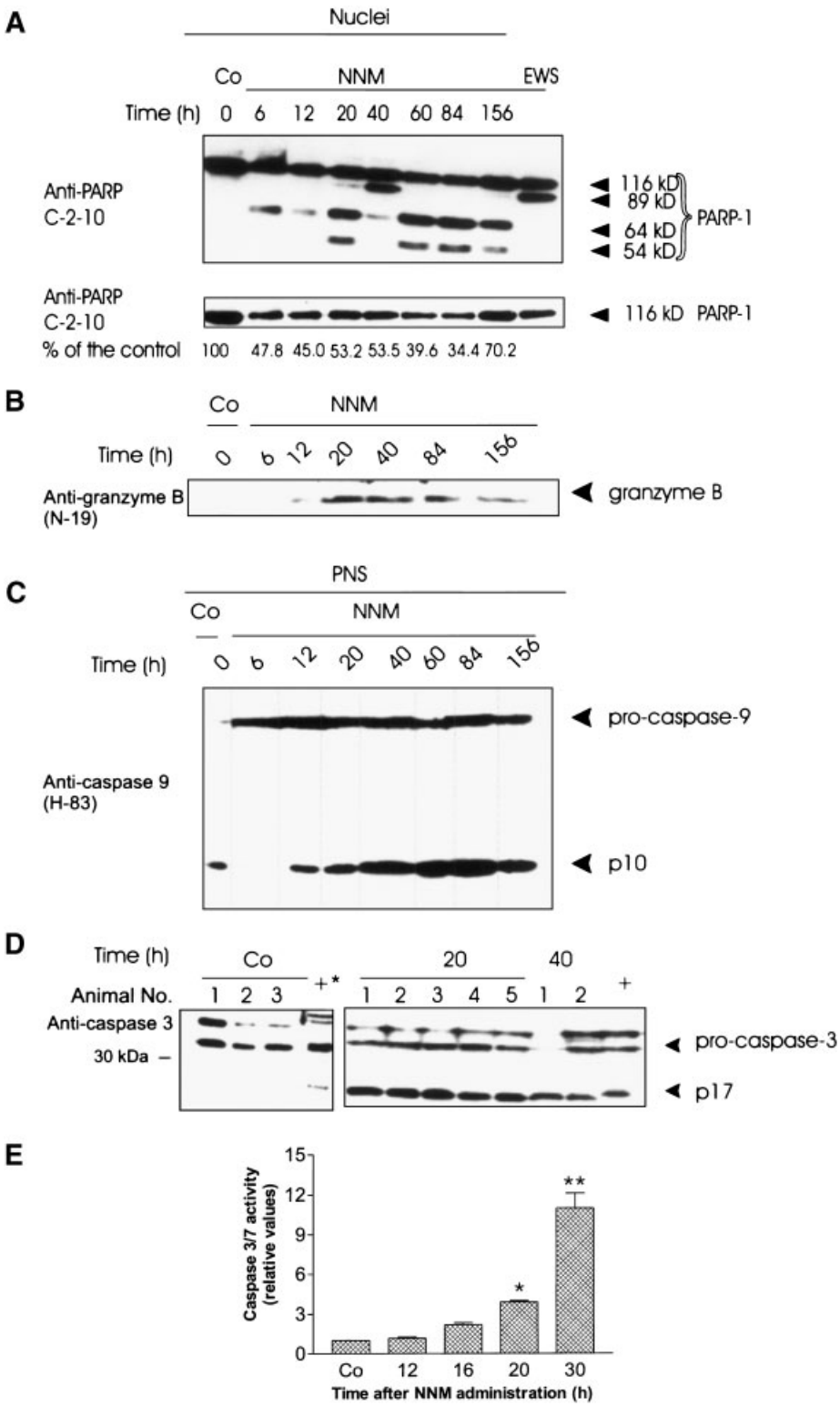
Fig. 1. Induction of apoptosis in rat liver after NNM treatment. **A**: NNM-induced fragmentation of DNA in rat liver. Tissue sections obtained from control rats and from animals at 12, 20, and 40 h after administration of NNM were used for TUNEL assay. Appearance of DNA strand breaks was assessed by TUNEL assay in situ, total DNA was visualized by propidium iodide staining. At sites where fluorescein-labeled damaged DNA overlapped with condensed chromatin, the yellow color was generated. Two different images at high magnification (objective 40×) from different areas of the section were obtained for control rats and at 12 and 20 h post-treatment. The image of preparation at 40 h was obtained at low magnification (objective 20×) to visualize global distribution of TUNEL positive nuclei. **B**: Incidence of apoptosis. Four thousands hepatocytes per rat per time-point were scored for the determination of the number of TUNEL positive hepatocytes. Only nuclei displaying concomitantly a TUNEL positive signal and condensed chromatin characteristic for apoptosis were scored for apoptosis. For each time-point tissue sections from one animal were evaluated.

at 40 h post-treatment strongly indicated that the effector caspases have been activated. Therefore, we performed immunoblotting analysis to assess the activation of caspases-9 and -3. Since caspases are primarily operating in cytoplasm, we analyzed proteins of post-nuclear supernatants obtained during subfractionation of rat liver samples from control animals and rats exposed to NNM. As shown in Figure 2C, no signal representing activated caspase-9 could be detected in a sample obtained 6 h post-treatment. At 12 h after NNM administration, a 10-kDa protein band representing the small subunit of activated caspase-9 appeared. The intensity of this band increased after longer time post-treatment. Examination of the caspase-3 status by immunoblotting (Fig. 2D) revealed its activation appearing 20 h after NNM administration. The strong p17 band representing the large subunit of activated caspase-3 was detected in samples obtained from five distinct animals. At 40 h post-treatment the intensity of activated caspase-3 decreased. In controls only the pro-zymogen form was detected. The activation of caspase-3 followed temporally that of caspase-9 and preceded the generation of a strong band at 89 kDa representing the canonical fragment of PARP-1.

The kinetics of caspase-3 activation after NNM administration was additionally evaluated using APO-ONE Caspase-3/7 homogenous assay. The basal activity of caspase-3/7 in control rats was very low (about 11,000 cpm). As shown in Figure 2E, the activity of caspases-3 and -7 in rat liver increased very slowly after NNM administration. At 12 h post-treatment no increase was observed. An onset of caspases activation (about twofold) was observed at 16 h post-treatment. At 30 h after administration activity of caspases-3 and-7 increased approximately 10-fold as compared to the untreated controls.

Release of Cytochrome C and AIF From Mitochondria

The depolarization of mitochondrial membrane induced by a variety of stress stimuli results in the release of distinct proteins into the cytoplasm. Release of cytochrome C is an early event during execution of apoptosis. In the next step we examined whether of cytochrome C was


A
Nuclei
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Time (h) 0 6 12 20 40 60 84 156
Anti-PARP C-2-10
116 kD
89 kD
64 kD
54 kD
PARP-1
Anti-PARP C-2-10
116 kD PARP-1
% of the control 100 47.8 45.0 53.2 53.5 39.6 34.4 70.2
B
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Anti-caspase 3
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15
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Time after NNM administration (h)

Fig. 2.

accumulated in cytoplasm samples prepared from liver of NNM-treated rats. First, we proved whether the accumulation of cytochrome C is detectable in crude post-nuclear supernatant. As shown in Figure 3A, a low level of cytochrome C detected in crude cytosol samples obtained from control rats markedly increased after NNM treatment. Liver homogenate and whole cell lysate (WCL) from HeLa cells were loaded as a positive control. The S-100 cytosol fraction isolated from untreated human HeLa cells was used as a negative control. The weak positive signals in samples obtained from control animals indicated that the crude post-nuclear supernatant contains residual mitochondria whereas the S-100 fraction of HeLa cells was devoid of them. Therefore, we additionally fractionated crude post-nuclear supernatant according to the method described by Fiskum et al. [2000]. During the ultracentrifugation step mitochondria were pelleted yielding a supernatant which was designated as a S-100 fraction. The analysis of S-100 samples revealed the time-dependent release of cytochrome C from mitochondria beginning 1 h after NNM administration (Fig. 3B). We examined also the cytoplasmic accumulation of another mitochondrial protein namely AIF. The release of AIF from mitochondria was slightly delayed as compared to that of cytochrome C. A first very weak AIF signal was detected in S-100 samples at 12 h after NNM treatment and increased at 20 and 30 h post-treatment (Fig. 3C).

Rapid Induction of p73 Protein in Response to NNM Treatment

Unlike the *p53 g*ene, the *p73* gene encodes multiple transcript isoforms. Therefore, suitability of different anti-p73 antibodies was tested. The specificity of distinct anti-p73 antibodies was proved by immunoprecipitation of in vitro translated human p73 isoforms (Fig. 4A,C). Anti-p73 antibodies specific for α and β forms obtained from different sources precipitated very efficiently both p73 ^{35}S-methionine labeled in vitro translated isomers (Fig. 4A). Furthermore, antibodies detected both p73 isomers in human cisplatin treated MCF-7 cells (positive control) and failed to react with any antigen in human Saos-2 cells that don't express p73 (negative control) (Fig. 4B**)**. On the other hand, the polyclonal antibody H-79 precipitated four distinct splicing variants of p73 (Fig. 4C).

To examine the effect of NNM treatment on the expression of p73 protein in rat liver, proteins of isolated nuclei were analyzed by immunoblotting using anti-p73 antibodies. p73

Fig. 2. Activation of caspases in rat liver after NNM treatment. **A**: Degradation of PARP-1. The appearance of PARP-1 fragments beginning at 6 h post-treatment coincided with the reduction of the full-length enzyme. The 89 kDa caspase-3 generated cleavage product of PARP-1 appeared at 20 h and increased markedly at 40 h. This correlated with the onset of the activation of caspas3/7 (**E**). Proteins of nuclei isolated from rat liver (20 μg/lane) were separated on 10% SDS-slab gels and transferred onto PVDF membrane. The blots were incubated with the monoclonal anti-PARP C-2-10 antibodies at a dilution of 1:3,000. After incubation with secondary antibodies linked to HRP diluted to a final concentrations of 1:20,000, immune complexes were detected by chemiluminescence. The monoclonal antibody C-2-10 recognizes the full-length enzyme as well as its carboxy-terminal cleavage products but does not react with the amino-terminal fragment generated by apoptotic proteases. A shortly exposed blot depicting a decrease of intact full-length PARP-1 (**lower panel**) was evaluated by densitometry. The intensity of the PARP-1 band in the untreated control was defined as 100%. A cell lysate of the human Ewing tumor cell line (EWS) expressing ts $p53^{138val}$ was loaded as a positive control of caspase-3 generated PARP-1 cleavage. In this cell line, PARP-1 is degraded by caspase-3 during the early stages of apoptosis [Kovar et al., 2000]. **B**: Accumulation of granzyme B in the hepatocyte nuclei of NNM-treated rats correlates with the generation of a 64 kDa PARP-1 fragment. Proteins of rat liver nuclei (20 μg/lane) were resolved on 10% SDS-slab gels. The blots were incubated with anti-granzyme B antibodies. **C**: Detection of activated caspase-9 by immunoblotting in rat liver at 12 h after NNM administration. The antibody reacted with the pro-zymogen and p10 protein representing the small subunit of activated caspase-9. Post-nuclear supernatant (PNS) was loaded on 10% gels. **D**: Activation of caspase-3 coincides temporally with the generation of the 89 kDa cleavage product of PARP-1. In the samples of post-nuclear supernatant prepared from control animals only the pro-enzyme form was detected. At 20 and 40 h after NNM administration p17 representing a large subunit of activated caspase-3 was additionally detected. At 20 and 40 h samples obtained from 5 and 2 distinct animals, respectively, were analyzed. Proteins (20 μg/lane) were separated on 15% gels. WCL (+*) or cytosol (+) of HeLA cells treated with cisplatin was loaded as a positive control. E: Quantitative evaluation of caspase activity in rat liver using the ApoOne Caspase-3/7. Cytosol samples were diluted with sucrose buffer and 50 μl of the sample was mixed with 50 μl caspases substrate Z-DEVD-R110. After incubation for 1 and 2 h at 37°C, the fluorescence was measured at 485 nm. Sucrose solution was used as a blank. BKG fluorescence was between 109 and 315 cpm. Assay was performed in duplicate; for each time-point at least two samples of two animals were determined. The graph was generated from values obtained after 2 h incubation. Statistical significance was determined using the ANOVA test followed by Dunnett's MultipleComparison test (all treatment groups vs. control). Bars indicate mean ± SD. Asterisks indicate the statistical significance valuated with ANOVA test: $^{**}P < 0.01$; $^{*}P < 0.05$.

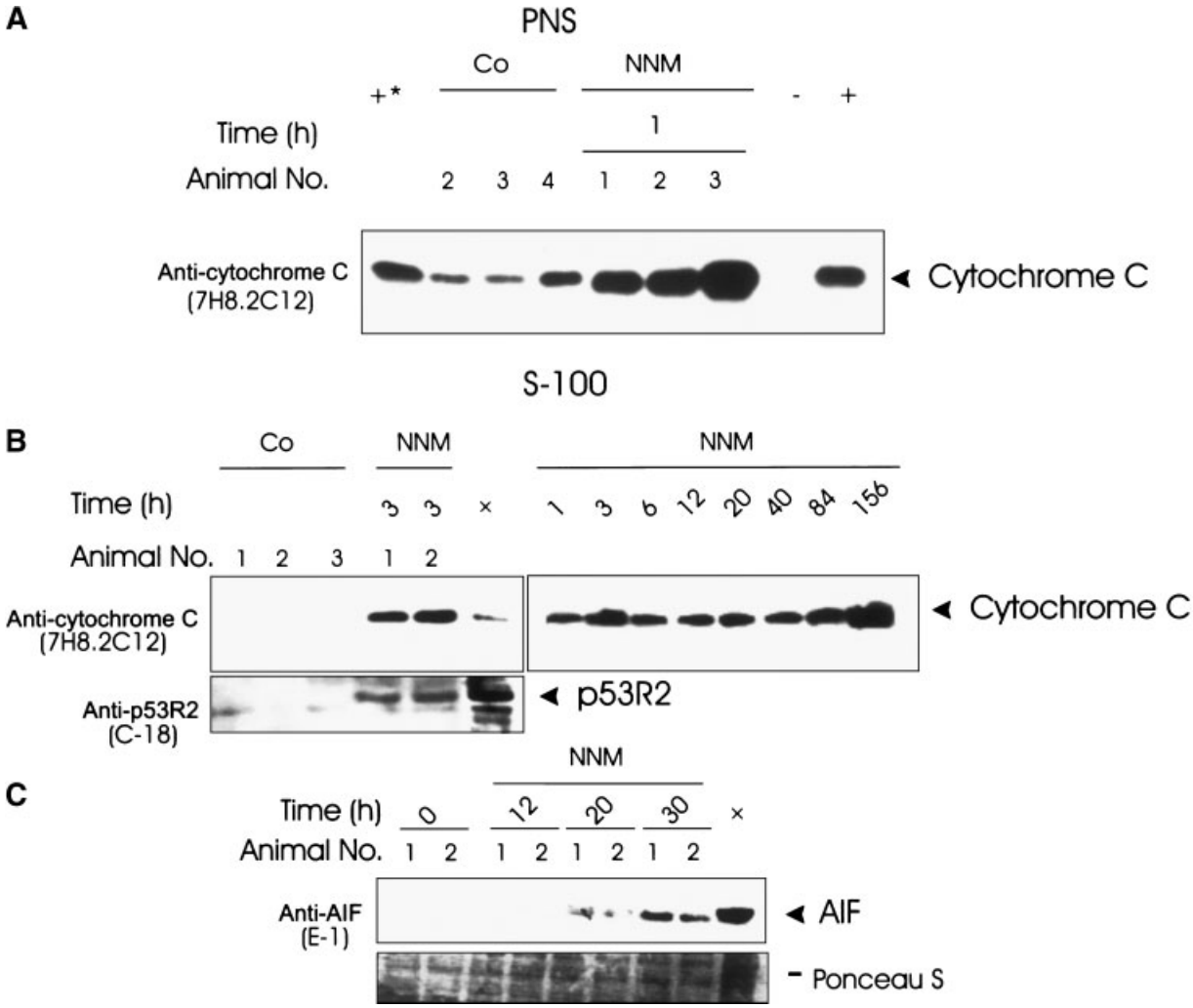

Fig. 3. Different kinetics of the release of distinct proteins from mitochondria after NNM administration. **A**: Strong accumulation of cytochrome C in crude cytosol isolated from rat liver 1 h after NNM. Proteins of post-nuclear supernatant were loaded on 15% SDS slab gel. **B**: Early release of cytochrome C into the cytoplasm in response to NNM treatment. No cytochrome C was detected in S-100 fraction prepared from control rats. Positive signal for cytochrome C appeared 1 h after NNM. **C**: Late accumulation of AIF in the cytoplasm of NNM-treated rats. Proteins of S-100 fraction prepared from rat liver were resolved on 10% SDS-slab gel. As a negative and a positive control, a S-100 fraction and WCL from untreated HeLa cells were loaded, respectively. Equal protein loading was confirmed by Ponceau S staining of the blot.

protein was barely detectable in liver nuclei isolated from untreated controls (Fig. 5). However, 6 h after NNM application p73 positive signals appeared in rat hepatocytes and reached a high level at 20 and 40 h post-treatment which persisted for at least 150 h (Fig. 5). Interestingly, two p73 splicing variants, α and β were induced by the hepatocarcinogen although with some variation in intensity. No additional p73 bands could be detected after incubation with anti-p73 antibody H-79 recognizing all p73 splicing variants (not shown). The above results were additionally confirmed by immunohistochemical staining. In liver sections obtained from untreated controls no p73 protein was detected in nuclei (not shown). At 12 h post-treatment numerous hepatocyte nuclei stained for p73 (Fig. 7). At the 20 and 40 h time-points, the number of p73 positive nuclei increased (not shown).

Different Kinetics of p53 and p73 Protein Activation

We have previously found by immunohistochemistry and by immunoblotting that the earliest detectable signals for p53 protein

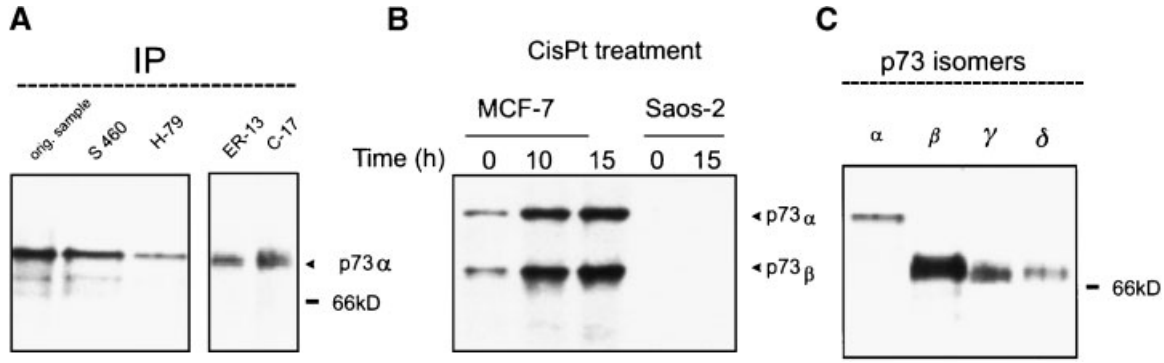

Fig. 4. Specificity of distinct p73 antibodies. **A**: Reactivity of distinct p73 antibodies with human recombinant p73 α protein. In vitro translated ^{35}S-methionine labeled human recombinant p73 α (input shown as an original sample) was precipitated by four distinct anti-p73 antibodies. Immune complexes purified by affinity chromatography on GammaBindG-Sepharose were analyzed on 10% SDS gels. Radioactively labeled proteins were detected autoradiographically after exposure of the blot against a MR Kodak film. **B**: Recognition of p73 isomers in human cells by polyclonal antibody S460. Nuclear proteins (20 μg/lane) obtained from control and cisplatin (CP) treated human cells (MCF-7 cells as a positive control and Saos-2 cells as a negative control) were separated on 10% SDS gels and immunoblotted with anti-p73 antibodies S460 (Abcam) at a final dilution of 1:2,000. **C**: Reactivity of polyclonal anti-p73 antibody H-79 with distinct human p73 recombinant isomers. Four in vitro translated ^{35}S-methionine labeled human p73 splicing variants were precipitated by anti-p73 polyclonal antibody H-79 (Santa Cruz, Santa Cruz, CA) recognizing all isomers. Immune complexes purified by affinity chromatography were electrophoretically separated and detected as described in panel A.

appeared in rat liver 24 h after NNM application. This finding was confirmed in this series of experiments. Unlike p73, p53 was induced in rat liver only transiently at 20 and 40 h post-treatment (Fig. 6). Remarkably, the extent of p53 induction was clearly weaker than that of p73. To exclude the possibility that p53 protein appeared earlier but was not detected by the monoclonal anti-p53 PAb421 antibody which does not recognize all forms of p53 throughout the cell cycle [Halazonetis et al., 1993], the blot was subsequently incubated with PAb240 antibody. This antibody reacts with a linear epitope located within the core domain of p53

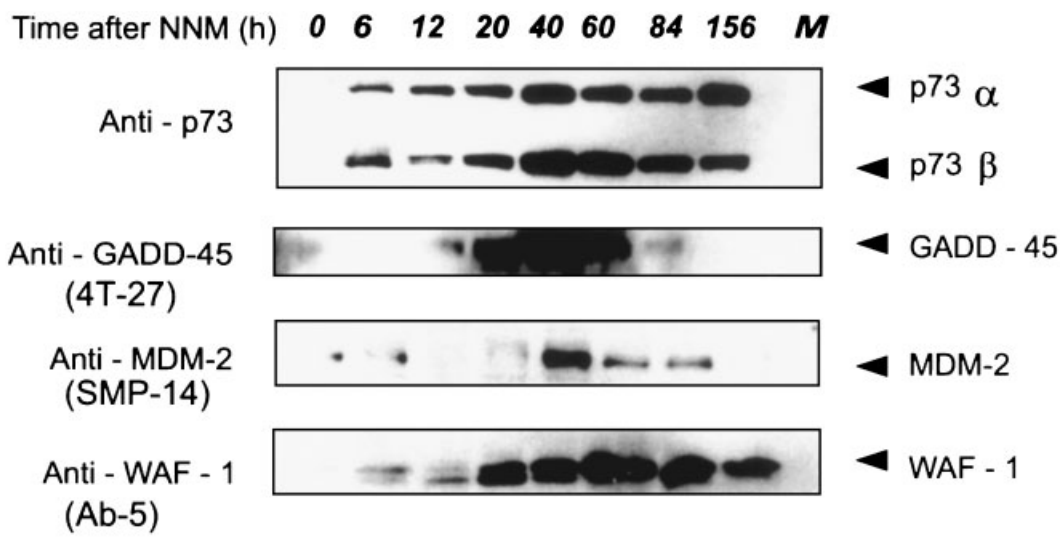

Fig. 5. Upregulation of p73 protein α and β and induction of target genes precede the onset of apoptosis in rat liver after NNM administration. Nuclear proteins (20 μg/lane) were separated on 10% or 15% (GADD-45 or waf-1) SDS-slab gels and transferred onto PVDF membrane The blots were incubated with corresponding primary antibodies diluted to a final concentration of 1:2,000 and subsequently with HRP-linked secondary antibodies in a final dilution of 1:10,000. M-Molecular weight markers (BSA: 66 kDa and carbonic anhydrase: 30 kDa) were loaded in the last lane). Positions of the molecular weight markers visualized by Ponceau S staining was indicative for the position of the reactive bands.

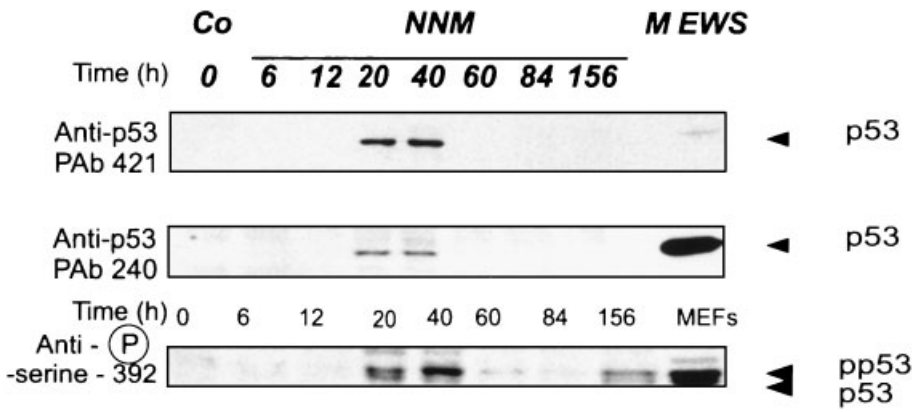

Fig. 6. Activation of p53 protein in rat liver 20 h after NNM administration. Nuclear proteins (20 μg/lane) were resolved on 10% SDS gels and transferred onto PVDF membrane. To discriminate between the latent and active p53 forms, the blot was sequentially incubated with monoclonal anti-p53 antibodies PAb421 and PAb240. A cell lysate of Ewing tumor cell line (EWS) exhibiting a latent p53 form was used as positive control. To identify phosphorylated forms of p53, the blot was incubated with a phospho-specific anti-p53 antibody. The immunoprecipitate from doxorubicin treated mouse embryo fibroblasts (MEFs) was used as a positive control.

protein and is known to recognize both latent and activated p53. As shown in Figure 6, PAb240 antibody detected a PAb421-non-reactive p53 form in a lysate of the Ewing tumor cell line overexpressing a temperature-sensitive $p53^{138val}$ mutant. However, the antibody did not reveal any additional p53 signal in the samples from NNM-treated rats. This indicates that no additional p53 form was present in rat liver nuclei at earlier time-points and that p53 protein detected at 20 and 40 h post-treatment represents its activated form. This was additionally substantiated by determination of phosphorylation of p53 and monitoring of DNA-binding activity (not shown). Immunoblotting using specific anti-phosphoserine-392 p53 antibody revealed the presence of two faint p53 bands 20 h post-treatment that merged to one strong band at 40 h. This indicates that full modification of p53 protein was not complete before 40 h and correlates temporally with the appearance of the capability of nuclear extracts to bind the p53 specific sequence as examined by EMSA (not shown).

Upregulation of p53/p73 Responsive Genes

It was of interest to study functional effects of the activation of p73 and p53 in rat liver. As expected, several downstream genes possessing the p53 consensus sequence in their promoters were induced. However, the onset of their upregulation differed. The earliest induction of GADD-45 and $p21^{waf-1}$ was observed 12 h after NNM administration and occurred 6 h later than the onset of p73 upregulation (Fig. 5). Interestingly, the stepwise upregulation of the target genes (low level at 12 h and higher level at 20 h) mimicked the stepwise increase of the p73 protein. On the other hand, MDM-2 protein, which is known to negatively regulate p53 transcriptional activity and expression, appeared at 40 h post-treatment. Subsequently, its concentration decreased at 60 h post-treatment to a barely detectable level despite of high levels of p73 protein. Thus, MDM-2 induction occurred approximately 30 h later than the p73 increase implicating that MDM-2 upregulation was mediated by p53 rather than by its bigger family member.

Early Apoptotic Hepatocytes Express p73 Protein

To evaluate the putative role of p73 in the induction of apoptosis, we examined the expression of p73 protein by immunohistochemistry in sections prepared from liver 12 h after NNM administration. A number of nuclei at early stages of apoptosis (Fig. 7) stained positively for p73 protein, thereby evidencing the coincidence between upregulation of p73 protein and induction of apoptosis.

Late Activation of Nuclear Factor NF-κB

To get better insights into the stress signaling in rat liver after NNM treatment in vivo, we determined the time course of the activation of NF-κB. Surprisingly, an accumulation of NF-κB in hepatocyte nuclei attributable to

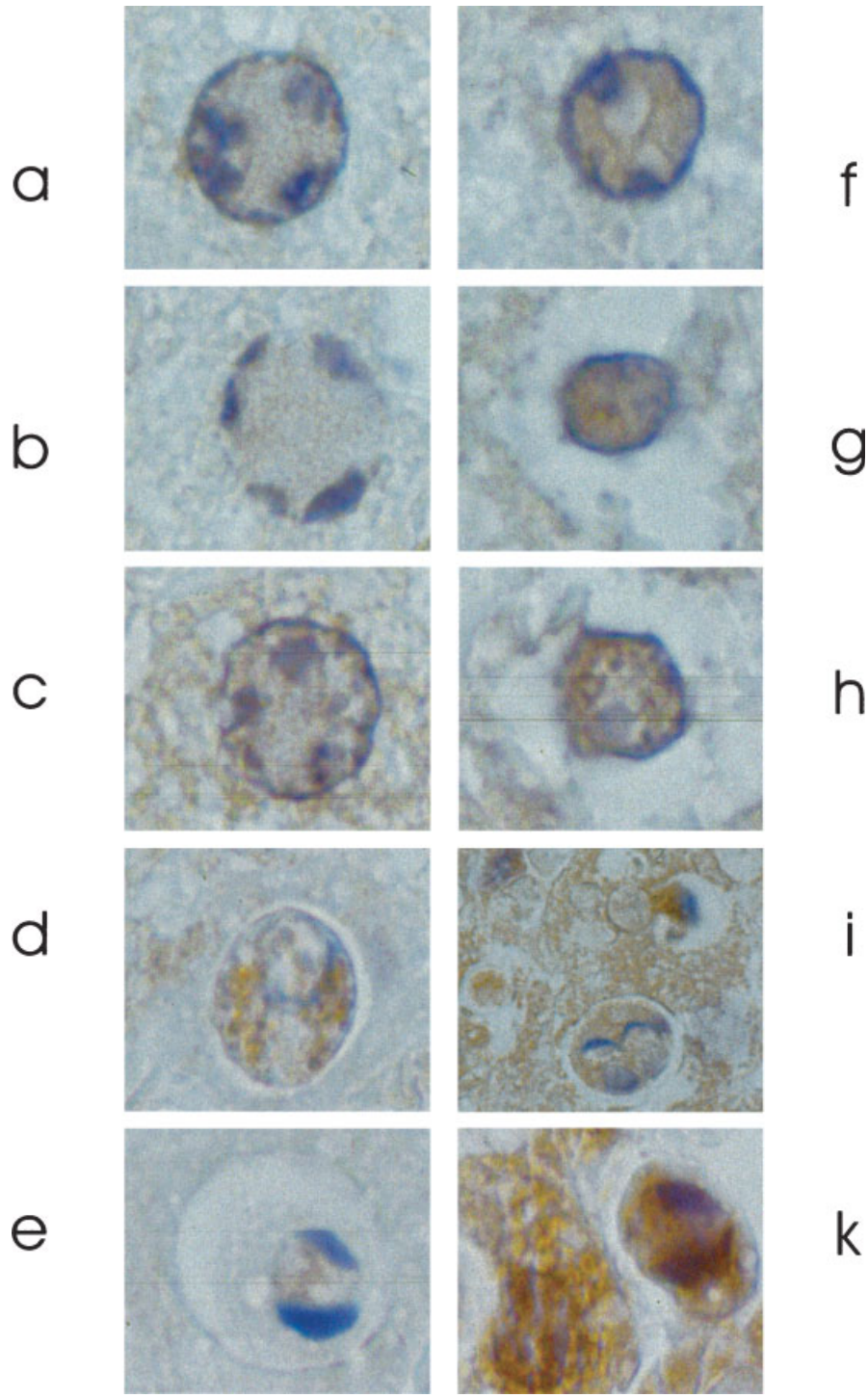

Fig. 7. Accumulation of p73 protein in the hepatocyte nuclei in early stages of apoptosis induced by NNM. **Panels a–k** depict hepatocyte nuclei at different stages of apoptosis at 12 h after NNM administration. p73-positive hepatocyte nuclei show brown immunostaining. Magnification: 150×. No p73 staining could be detected in tissue samples prepared from control rats (not shown).

translocation of the activated nuclear factor became evident already 20 h post-treatment (Fig. 8) and reached the highest level after another 20 h. This strictly correlated with an appearance of specific DNA binding activity as evidenced by EMSA (not shown) and was accompanied by a decrease of the levels of cytosolic IκB protein (Fig. 8). The translocation

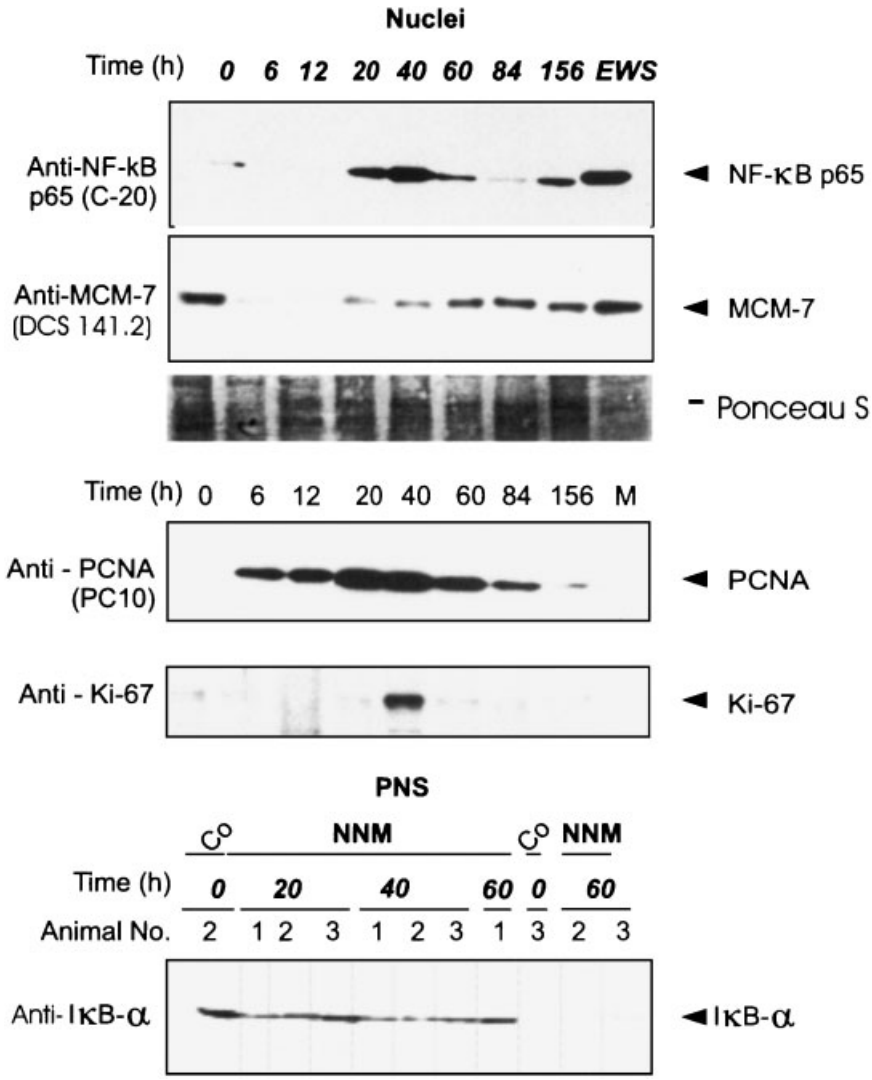

Fig. 8. Activation of stress-signaling protein NF-κB in rat liver 20 h after NNM administration. Nuclear accumulation of NF-κB correlated with the reduction of IκB level in cytosol. Nuclear proteins or cytosolic proteins (PNS) (20 μg/lane) were resolved on 10% (or 8% for Ki-67) SDS gels. Equal protein loading was confirmed by Ponceau S staining of the blot.

of NF-κB from the cytosol into the nuclei was additionally confirmed by immunohistochemistry (not shown).

Induction of Factors Involved in DNA Repair in the Absence of Detectable p53 Protein

To assess the other downstream effects exerted by upregulated p73 protein, we checked the expression of selected proteins known to be involved in DNA repair. In samples obtained from control rats no signal for p53R2 was detected (Fig. 3B). Positive p53R2 signals appeared in cytosol samples from rats 3 h after NNM administration. PCNA, which is transcriptionally activated by p53 was also enhanced after NNM treatment. The nuclear level of PCNA was upregulated in two steps (Fig. 8). PCNA concentration increased at 6 h and reached the highest level at 20 and 40 h post-treatment. Since PCNA is a component of the DNA replication and repair machinery, we additionally examined the expression of Ki-67 protein. A strong Ki-67 positive signal was detected at 40 h (Fig. 8). The strong upregulation of GADD-45 protein in the nuclei beginning at 20 h (Fig. 5) correlated strongly with the highest PCNA level. In contrast to these changes, the moderate level of the MCM7

protein in the nuclei isolated from liver of control rats transiently decreased after NNM administration (Fig. 8) and returned to the original status at 60 h. This comparison additionally substantiates the specificity of the observed changes of the expression of distinct cellular proteins after NNM administration.

DISCUSSION

Apoptosis is an essential physiologic multistep process necessary to appropriately eliminate damaged or initiated preneoplastic cells, thereby protecting organisms against tumor formation [Tamm et al., 2001]. Moreover, chemotherapeutic strategy based on application of drugs causing DNA damage is thought to kill cancer cells by apoptosis. However, resistance of tumor cells to chemotherapy remains an obstacle to the successful treatment of human malignancies. Therefore, the elucidation of mechanisms responsible for initiation and execution of apoptosis either in tumor protection or chemotherapy is of great importance. The tumor suppressor protein p53 has been reported to frequently mediate apoptosis after DNA damage in several cell types [Harris, 1996]. However, it is fundamentally important to consider the tissue context, when evaluating the roles of p53 in prevention of carcinogenesis. The involvement of p53 in the control of proliferation and apoptosis was thoroughly investigated by comparison of wt and p53 deficient mice [Bellamy et al., 1997]. The inactivation of *p53* gene disturbed the control of the proliferation and survival of the hepatocytes but did not reduce the apoptotic potential after genotoxic stress indicating that the relationship between p53 and the cellular response to stressors is more complex.

Recently, we showed that NNM-induced apoptosis in rat liver was p53-independent [Wesierska-Gadek et al., 1999]. The induction of apoptosis preceded by a few hours the activation of p53 protein. Twenty-four hours after NNM administration nuclear accumulation of p53 protein was observed by immunoblotting and by immunohistochemistry. The upregulation of p53 was attributable to the enhanced p53 transcription rate which preceded the appearance of p53 in the nuclei by 12 h [Wesierska-Gadek et al., 1999]. No p53 positive signals could be detected in the nuclei of hepatocytes undergoing apoptosis indicating the lack of direct p53 involvement in the induction of the apoptotic process. Surprisingly, despite a strong tissue injury apparent a few hours after NNM application, the intensity of p53 response activated in vivo after NNM administration was moderate and lower than expected [Wesierska-Gadek et al., 1999].

To explore the pathway of the p53-independent cell death after NNM we investigated whether it was mediated by p73, a p53-related protein. The recent data on inducibility of p73 in response to drugs causing DNA damage are contradictory. Earlier studies attested the lack of p73 induction after treatment with DNA damaging agents [Kaghad et al., 1997; Fang et al., 1999], whereas more recent investigations revealed that p73 protein is activated in cultivated cells in response to cisplatin and γ-radiation and subsequently initiates apoptosis [Agami et al., 1999, Gong et al., 1999, Yuan et al., 1999].

Our present data demonstrate that p73 protein is strongly activated in rat liver by NNM, a potent cytotoxic and genotoxic hepatocarcinogen. The nuclear accumulation of p73 protein in the liver beginning 6 h after NNM administration reached the highest level at 40 h and persisted for a few days. The onset of p73 induction preceded the increase of the apoptosis rate by 6 h. Beginning from 12 h post-treatment the rate of cell death was elevated. Moreover, several hepatocytes exhibiting morphological characteristics of early stages of apoptosis at 12 h post-treatment accumulated p73 protein in the nuclei as demonstrated by immunohistochemical staining. This coincidence is indicative for the involvement of p73 protein in the induction and/or execution of apoptosis. The examination of the kinetics of depolarization of mitochondria and activation of caspases revealed that the both processes were coordinated with different kinetics. The disruption of the mitochondrial transmembrane potential and the induction of granzyme B occurred earlier than the activation of caspase–cascade. The release of cytochrome C from mitochondria preceded for a few hours the accumulation of AIF in the cytoplasm. AIF is a ubiquitously expressed flavoprotein residing in the mitochondrial intermembrane space like cytochrome C [Daugas et al., 2000, Joza et al., 2001]. In response to a variety of death stimuli, AIF is released from mitochondria and accumulates in the cytoplasm resulting in loss of

asymmetric distribution of phosphatidylserine residues and subsequently translocates into the nucleus, where it induces the condensation of chromatin and large scale DNA fragmentation. In contrast to cytochrome C, which after complexing with APAF-1 contributes to the formation of the apoptosome and activation of caspases, AIF acts in a caspase-independent fashion. The activation of caspases was transposed in time and began 20 h after NNM administration. Concerning the role of granzyme B in the modulation of apoptotic events in rat liver after NNM administration, its possible contribution to the caspase activation is not quite clear. There are contradictory reports on the processing of caspase-3 by granzyme B. Some of them indicate that caspases-3 cannot be fully activated by granzyme B. It seems rather that activated granzyme B cleaves directly PARP-1 generating the carboxy-terminal fragment of 64 kDa. Our present results evidence clearly that caspases-3 was strongly induced in rat liver but not before 20 h after NNM administration. The activated caspases-3 generated the canonical 89 kDa degradation product of PARP-1. This observation seems to be contradictory to the previous observation of Jones et al. [1999] reporting that PARP-1 is not cleavable by caspases in isolated hepatocytes. However, considering the fact that cellular responses to distinct stress stimuli induced in whole organisms in vivo and in isolated cells are not directly comparable, it becomes clear that no discrepancy exists between the latter report and our findings. Moreover, even under in vivo conditions distinct animals exhibit different susceptibility to the action of cytotoxic agents. Thus, 40 h after NNM administration the caspase-3 mediated processing of PARP-1 occurred not in all tested animals. This clearly evidences that in response to the same insult different processing outcomes can be initiated.

It becomes obvious that not only apoptosis but also DNA repair was induced in the liver of rats exposed to NNM. Three distinct proteins involved in a p53/p73-inducible response to DNA damage: p53R2, GADD-45, and PCNA were upregulated. p53R2 shows striking similarity to the ribunucleotide reductase small subunit R2, which is important for DNA replication. p53R2 contains a p53-binding sequence in intron 1. Expression of p53R2 but not R2 is induced by a variety of genotoxic stresses in a p53/p73 dependent manner [Nakano et al., 2000; Tanaka et al., 2000]. An upregulation of p53R2 in liver cytoplasm was detected 3h post-treatment. Moreover GADD-45 and PCNA were also strongly induced in the nuclei of NNM-treated animals. PCNA has a dual function in the metabolism of nucleic acids [Bravo et al., 1987]. First, it plays an essential role in DNA replication as the auxillary protein of DNA Polymerase δ, the enzyme acting during the replication of chromosomal DNA [Bravo and Macdonald-Bravo, 1987]. DNA Polymerase δ complexing with PCNA is responsible for leading strand DNA synthesis. In quiescent cells there are very low levels of PCNA mRNA and protein. Upon mitogen stimulation of cells, there is a several fold increase of the PCNA transcription and PCNA protein accumulates in the nuclei shortly before DNA synthesis. The lack of PCNA observed in the nuclei of control rats is not surprising because the liver of adult rats is a mostly quiescent tissue. Secondly, PCNA is involved in the DNA excision repair [Shivji et al., 1992] and is required for the conversion of nicked intermediates to completed repair events [Shivji et al., 1992]. Regarding the dual function of PCNA, we additionally determined the expression of Ki-67 antigen as a criterion of replicative activity. A strong upregulation of Ki-67 antigen found at 40 h post-treatment coincided temporally with the onset of regenerative DNA synthesis [Wesierska-Gadek et al., 1999], thereby indicating that the PCNA increase observed at 20 h and before is rather attributable to the DNA repair. Interestingly, a strong PCNA accumulation in the nuclei at 20 h post-treatment correlated with the activation of GADD-45. This temporal coincidence may be of functional importance because both proteins are known to interact [Smith et al., 1994]. GADD-45 was found to inhibit the entry of stressed cells into S-phase what establishes a link between the p53/73 dependent growth suppression and DNA repair. These results are in concordance with recent observation describing the involvement of p73 protein in DNA repair [Shimodaira et al., 2003].

Unlike p73, p53 response in liver to NNM treatment in vivo was markedly delayed and significantly weaker. Activation of p53 can be modulated not only by either enhanced transcription/translation or by elongated stability but also by a conversion from a latent to an active form of p53. These two p53 forms differ with respect to subnuclear distribution [Rubbi

and Milner, 2000], DNA-binding activity, and reactivity with the anti-p53 antibody PAb421 [Halazonetis et al., 1993]. There is indirect evidence that the latent p53 form responds preferentially to damage of actively transcribed genes [Yamaizumi and Sugano, 1994]. The discovery that nucleoplasmic and nucleolar p53 is associated with the site of transcription might explain how few p53 molecules can efficiently detect DNA damage. Moreover, it provides also information that the damage of actively transcribed genes preferentially triggers p53 response. In light of this information, it is not surprising that the p53 response to the hepatocarcinogen NNM in rat liver is moderate.

The biological role of p73 became evident especially in cells lacking p53 (e.g., Saos-2 or p53−/− MEFs) or in E6 expressing cells. However, such exact dissection of the activities exerted by induced p53 and p73 proteins is difficult in experiments performed in vivo with normal, genetically non-manipulated animals. To explore the functional consequences of the upregulation of p73 α and β and to discriminate between p53- or p73-driven effects, the expression of some downstream genes was examined. The onset and kinetics of stimulation of GADD-45 and $p21^{waf1}$ in rat liver coincided with that of p73 protein. Expression of both proteins was 6 h delayed as compared to p73. However, MDM-2 protein appearing only transiently with a maximum at 40 h post-treatment mimicked the kinetics of p53 induction with some retardation.

These results are consistent with recent findings describing a significant difference between the mechanism that govern the transcriptional activity of p53 and p73 [Lee and La Thangue, 1999]. It has been shown that p73 activates GADD-45 more efficiently than p53 [Lee and La Thangue, 1999], whereas the reverse situation was apparent for the MDM-2 gene [Lee and La Thangue, 1999]. A strong NNM-induced p73 response in liver tissue exceeding that of p53 raises the question whether both related genes, p53 and p73 can reciprocally impair their expression and function. A very recent study [Vikhanskaya et al., 2000] showed that hyperexpressed p73 reduced endogenous p53 transcriptional activity and competed for p53 DNA binding. It would suggest that p73 can modulate p53 function and that overexpression of p73 in tumors might be a novel mechanism of inactivation of p53. On the other hand, it has been shown that p63 and p73 are required for p53-dependent apoptosis after DNA damage [Flores et al., 2002]. This latter report would implicate that both related proteins p73 and p53 interact and cooperate [Urist and Prives, 2002]. However, the role of p73 in carcinogenesis remains ambiguous at present. The *p73* gene has been mapped to 1p36.3 [Kaghad et al., 1997], a region that is frequently deleted in many cancers including neuroblastoma, colorectal cancer, and breast cancer. In contrast to p53, mutations in the coding sequences of p73 are rare in human malignancies [Ikawa et al., 1999]. However, contrary to the first study reporting monoallelic expression of p73 in some tissues, its biallelic expression in normal lung was demonstrated. Unexpectedly, several groups showed the activation of the silent p73 allele in prostatic, colorectal, lung, and kidney tumors [Ikawa et al., 1999]. Recently, upregulation of both p73 α and β was found in a subset of hepatocellular carcinomas [Herath et al., 2000]. One of the most intriguing observations was that enhanced p73 expression tended to occur in HCC cases with loss of heterozygosity of p53. It would imply that inactivation of p53 appears to be associated with upregulation of p73 expression.

In conclusion, the present work demonstrates for the first time that p73 is strongly induced in rat liver by the hepatocarcinogen NNM and that upregulated p73 accumulated in the nuclei of hepatocytes in early stages of apoptosis. Moreover, it shows that both related genes, p73 and p53 differ in the onset and the kinetics of the activation and duration of their response. On the basis of present data, we cannot exclude that p73 competes and interferes with the p53 protein.

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