Characterizing the role of MDM2 in diethylnitrosamine induced acute liver damage and development of pre-neoplastic lesions

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Pre-neoplastic lesions in rodent liver often express high levels of MDM2 and lack a p53 response to DNA damage. The question we posed was whether there is a liver-specific regulation of the p53/MDM2 feedback loop and if it can be related to the development of pre-neoplastic lesions, referred to as enzyme altered foci (EAF) in rats. Acute responses of p53 and MDM2 to diethylnitrosamine (DEN) were characterized by employing immunohistochemistry, western blotting, RT±PCR and in situ hybridization. A single dose of DEN induced a centrilobular p53 response that peaked at 24 h. It was associated with transcriptional activation of MDM2 and signs of apoptosis. However, in midzonal hepatocytes, which constitutively expressed high levels of cytoplasmic MDM2, there was a rapid-onset but transient p53 response. It was terminated at 24 h and there were no signs of apoptosis. The rapidly declining p53 levels in midzonal areas was preceded by a transient peak in MDM2 mRNA levels at 6 h. Rats pre-treated with repeated low or high weekly doses of DEN exhibited EAF and these lesions expressed high levels of cytoplasmic MDM2. Using MDM2 as a marker for EAF gave similar results as using glutathione transferase-P (GST-P) as a marker. Furthermore, small EAF, elicited by low doses of DEN, were preferentially localized to midzonal areas. It is concluded that in centrilobular areas DEN-induced alterations in p53/MDM2 levels are compatible with a previously described feedback loop. An attenuated p53 response in midzonal hepatocytes can be related to a high constitutive expression of MDM2 in these cells. The localization of small EAF to midzonal areas, and the fact that EAF cells expressed high levels of MDM2, indicates that EAF expression is a factor governing initiation and early development of EAF. The data support the hypothesis that EAF hepatocytes are initiated via epigenetic mechanisms.

Introduction

Nuclear levels of the sequence-specific transcription factor and tumor suppressor p53 are controlled by an autoregulatory loop (1,2). Stressful stimuli, including different types of DNA damages, may induce accumulation of p53 and subsequent transactivation of the MDM2 gene. The product of the MDM2 gene is a p53 ubiquitinase and accumulation of this protein in the nucleus leads to export and proteolytic degradation of p53. Under certain conditions, oscillations rather than a single pulse, in the levels of p53 and MDM2 might determine downstream events (3).

Post-translational modifications of the p53 protein, including phosphorylations in response to DNA damage, appear to be important events in stabilizing and activating p53 (4–6). Certain of the kinases responsive to DNA damage may also phosphorylate MDM2, possibly alleviating inhibitory effects on p53 (7–9). Some DNA-damaging agents may stabilize p53 by down-regulating transcription of MDM2 (10).

Stresses associated with in vitro systems may affect feedback control of the level of p53 and its function (11), and we know of no study characterizing the p53/MDM2 loop in vivo in hepatic tissue. It has been suggested that rodent liver may exhibit tissue-specific regulation of p53 (12). This suggestion originates from the finding that the p53 response to ionizing radiation is weak in mouse and rat liver (13–15). In contrast, the genotoxic food contaminant and liver carcinogen diethylnitrosamine (DEN) induces a clear p53 response, seen in centrilobular areas 24 h after DEN administration (16).

Many putative pre-neoplastic focal lesions, i.e. glutathione transferase-P (GST-P)-positive lesions often referred to as enzyme altered foci (EAF), do not accumulate p53 in response to DNA damage (17–20). They also express high levels of MDM2 (20). The fraction of EAF exhibiting an attenuated p53 response increased with increasing treatment time and become the dominating phenotype after 10–12 weekly treatments with DEN. However, phenobarbital-treated rats did not exhibit EAF with an attenuated p53 response. These results suggest that, under conditions of genotoxic stress, the attenuated p53 response may confer growth advantages to affected lesions (18).

EAF develop in very large numbers in rat liver, and mutations in, for example, the p53 gene are rarely detected in such lesions (21). Thus, random mutational changes may not readily explain the attenuated p53 response. Instead, our (17–19) and other data (22,23) have been interpreted to indicate that EAF hepatocytes are initiated via epigenetic mechanisms. Interestingly, recent data suggest that p53 is antagonized and down regulated by c-Jun and that this effect is crucial for the promotion of DEN-induced mouse liver tumors (24). Earlier studies show that c-Jun may be activated by alterations in the methylation status of the gene (25), so epigenetic alterations may explain both c-Jun activation and the attenuated p53 response to DNA damage.

In the present study we have explored the acute in vivo response to DEN further in order to understand mechanisms leading to EAF development. We have also analyzed EAF in rat livers and present data regarding EAF localization in the liver lobule. The question we posed was whether the altered p53 response in EAF could be related to perhaps a liver-specific regulation of the p53/MDM2 response in normal liver tissue. We find that midzonal hepatocytes exhibit a...
constitutively high expression of MDM2 and that these cells have a curtailed p53 response to a single dose of DEN. We also find that EAF hepatocytes express high levels of cytoplasmic MDM2 and that a majority of EAF, induced by repeated low doses of DEN, are localized in midzonal areas of the liver lobule.

Materials and methods

Treatment of the donor animals

Female Sprague-Dawley rats (180 g) were injected intraperitoneally (i.p.) with a single marginally toxic dose of DEN (Sigma, St Louis, MO) (0.99 mmol/kg body wt in the case of immunohistological staining and in situ hybridization (ISH); and 1.32 mmol/kg body wt for immunoprecipitation/western blotting). Two to five animals were killed at each interval (0–72 h). In a low dose EAF experiment (Table III), three adult female Sprague-Dawley rats were injected i.p. with 10 repeated doses of DEN (25 mg/kg). We also re-examined rats from earlier EAF experiments (Table II). Four groups of rats, all initiated with DEN neonatally, were given two or 10–12 weekly subtoxic doses (0.33 mmol/kg body wt) of DEN, or phenobarbital (500 p.p.m. in the drinking water) for 3 or 14 months (18). All EAF bearing animals received a challenging dose of DEN (0.99 mmol/kg body wt) 24 h before death (17).

Immunohistological staining

Livers were fixed and slices were stained as described previously (16,17). In brief, livers were perfused with 3.7% buffered formaldehyde for 1.5 h and subsequently placed in formalin for 24 h. The slides stained for p53, MDM2 and PCNA were treated with microwaves five times for 5 min each time prior to staining. In case of double staining for p53 and GST-P the sections were incubated overnight with primary antibodies. Secondary alkaline phosphatase-conjugated anti-rabbit antibodies were then applied to the GST-P slides. Incubation continued for 1.5 h. DAKO® Fuschin + Substrate-Chromogen System (DAKO, Denmark) was employed to detect GST-P, while p53 was visualized using the EnVision™ peroxidase kit (DAKO) with 3-diaminobenzidine tetrahydrochloride as substrate. Other primary antibodies were visualized using the EnVision™+ peroxidase kit. Antibodies used were rabbit anti-GST-P (Biotrin, Ireland), mouse monoclonals SMP-14 (Santa Cruz, Santa Cruz, CA), 2A10 (Oncogene™, Cambridge, MA) towards MDM2, pAb 240 towards p53 (Oncogene), PC10 for PCNA (Calbiochem, San Diego, CA) and rabbit anti-CYP 2E1 (kindly provided by Dr Magnus Ingelman-Sundberg). The TdT FragEL™ apoptosis kit (Calbiochem) was used for TUNEL (Terminal Deoxynucleotidyl Transferase-Mediated X-dUTP Nick-End Labeling) staining.

Phosphatase treatment was similar to the one reported for western blotting (26). In brief, endogenous peroxidases were first inhibited by incubation with 3% H2O2 in methanol for 30 min. The slides were subsequently incubated with 5 U CIAP for 1 h at 37°C, following which this enzyme was inhibited by incubation in EGTG at 65°C for 10 min. Thereafter, slides were placed into 2.5% BSA in TBS for 30 min and then exposed to primary antibodies. Finally, the MDM2–antibody complex was visualized employing the EnVision™+ peroxidase kit.

Analysis of immunohistologically stained liver sections

Stained liver sections were analyzed microscopically. The minimum section area analyzed per liver was 40 mm² and all GST-P-positive EAF within a section with a size ≥0.0009 mm² were analyzed. Calculation of EAF parameters was performed using software written by Peter et al. (27). Localization of EAF was determined by counting all MDM2-positive EAF followed by classification dependent on their zonal localization.

Immunoprecipitation and western blotting

Livers were homogenized and subfractionated. Immunoprecipitation of p53 was then performed as described previously (19), using monoclonal pAb122 antibodies (Bohreinger Mannheim, Germany) and protein A-Sepharose (Pharmacia, Uppsala, Sweden). MDM2 was immunoprecipitated with the polyclonal H-221 antibody (Santa Cruz) and protein A/G agarose (Santa Cruz). p53 was detected with CM-1 polyclonal antibodies (Novocastra Laboratories, Newcastle, UK) and with rabbit antibodies directed against p53 phosphorylated at the residues Ser-15, -20 and -392 (New England Biolabs, Beverly, MA). MDM2 was immunoprecipitated with the anti-GST-P (Biotrin, Ireland), mouse monoclonals SMP-14 (Santa Cruz, Santa Cruz, CA). 2A10 monoclonal antibodies (Oncogene™) directed towards MDM2 were also employed. The latter was employed in connection with antigen demasking by phosphatase treatment as described by Maya and Oren (26). Visualization of precipitated proteins was achieved as described previously (17).

RT–PCR analysis

Livers from the rats were removed and submerged in RNAlater™ (Ambion, Austin, TX), after which RNA was isolated using the Totally™ RNA kit (Ambion). The RNA thus obtained was analyzed employing multiplex comparative RT–PCR analysis using the QuantumRNA kit, alternate 18S Internal Standard (Ambion) in combination with the Qiagen™ OneStep RT–PCR kit (Qiagen, Valencia, CA).

The optimal 18S primer/18S competitor ratios were determined empirically to be 1/9 for p53, 2/8 for MDM2, 2/8 for p21 and 3/7 for GAPDH. The primer sequences for each of these were as follows: p53, forward, 5′-TCC TTC CCA ACA TCA TAT CC-3′; reverse, 5′-GCA CCA ACA AGC ACC TCA AA-3′ (18); MDM2, forward, 5′-TGT GCA ATA CCA ACA TGT CTG-3′; reverse, 5′-CTC CAA TAG TCA GCT AAG G-3′ (29); p21, forward, 5′-GAT CCA CAG CAT TGA GA-3′; reverse, 5′-ATA CTG TTA GGC TGG TCT GC-3′ (30). And finally, GAPDH, forward, 5′-GGT GCT GAT TGC GTG GA-3′; reverse-5′-GCC ATG CCA GTG AGC TTC CC-3′ (31). The sizes of the RT–PCR products obtained were: 261, 910, 450 and 243 bp, respectively. The MDM2 gene has two promoters, P1 and P2, in humans and mice (11) whereas the rat gene has not been characterized in this regard, and the primers used do not discriminate between these putative transcripts. We have tested (under more or less stringent conditions) mouse primers specific for P1 and P2, respectively, but obtained no signals in our rat samples.

Reverse transcription was performed at 50°C for 30 min. The annealing temperatures were 55 (p53), 52 (MDM2), 64 (p21) and 50°C (GAPDH). The other cycling parameters were set according to the manufacturer’s instructions. RT–PCR products were separated on a 1.5% ethidium bromide-containing agarose gel. For samples within the linear range of amplification, band density ratios were calculated in relationship to the intensity of the band containing the internal standard 18S.

ISH

In order to generate a template for in vitro transcription, total RNA was first isolated from rat liver (as above) using the Totally™ RNA kit (Ambion). Two micrograms of this RNA was subjected to a one-step RT–PCR reaction (Qiagen, Valencia, CA) involving primers specific for MDM2 (described above). A core T7 phage promoter was added onto the MDM2 reverse primer, in order to generate an antisense template for subsequent in vitro transcription utilizing the MaxiScript kit (Ambion). The probe was labeled with Diogenixin-UTP (Roche, Mannheim, Germany) during the process of in vitro transcription and thereafter hydrolyzed in a Na2CO3 buffer at pH 10.2. Unincorporated nucleotides were removed using Sephadex G-50 mini Quick Spin RNA Columns (Roche, Mannheim, Germany). Sections were treated and probed as described previously (32).

Results

Immunohistochemistry

In control experiments involving untreated rats no staining for p53 was detectable. However, employing an anti-MDM2 antibody (SMP14) resulted in weak cytoplasmic staining in the majority of hepatocytes (Figure 1A and summarized in Table I). There was also a layer of four to five cells that demonstrated stronger cytoplasmic staining. This zone often clearly delineated by the more heavily stained cytoplasm. No staining was seen when the SMP14 antibody, or any other of the used antibodies, was omitted.

We also stained control livers for CYP2E1, which is the major cytochrome P450 isozyme involved in phase 1 activation of DEN (33). The stained areas were similar to those shown in rats treated with DEN 3 h prior to death (Figure 1B) and partially overlapped those with a strong cytoplasmic staining for MDM2.

Rats were treated with DEN and killed 3–72 h later. Three hours after DEN treatment no nuclear staining for p53 or MDM2 (SMP14) was detected (not shown). However, as indicated in the introduction, MDM2 can be phosphorylated in response to DNA damage, and we tested whether pretreatment of sections with CIAP [a phosphatase shown to
unmask phosphorylated epitopes on MDM2 and thereby increase the immunoreactivity of this protein (26), enhanced antibody recognition. We found that CIAP markedly increased the nuclear staining with the 2A10 antibody (directed against residues 258±260 and 393±395) in sections from rats treated with DEN 3 h prior to death (Figure 1C) but not at earlier time-points. Positive nuclei were evenly distributed between the centrilobular and midzonal areas staining positively for CYP2E1. Without CIAP there was no nuclear staining (not shown). The 2A10 antibody also produced cytoplasmic staining in midzonal areas of livers of untreated rats (not shown), which overlapped the staining obtained with the SMP14 antibody.

Six hours after DEN treatment a partial loss of the midzonal cytoplasmic staining was seen with the SMP14 antibody for MDM2 (Figure 1D) and a nuclear staining in midzonal and

**Table I. Changes in MDM2 and p53 expression in hepatic zones following a single dose of DEN**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Periportal area</th>
<th>Midzonal area</th>
<th>Centrilobular area</th>
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<tbody>
<tr>
<td></td>
<td>p53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MDM2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MDM2 mRNA&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>N</td>
<td>C</td>
<td>N</td>
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<td>3</td>
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<td>12</td>
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<tr>
<td>24</td>
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N, nuclear staining; C, cytoplasmic staining. Empty space, not analyzed; −, no signal.

<sup>a</sup>As assessed by IHC (SMP-14) and western blotting.
<sup>b</sup>As assessed by ISH and RT±PCR.
<sup>c</sup>ISH (CIAP-pre-treatment, 2A10).
<sup>d</sup>Morphologically different from that seen at 0 and 3 h (see text).
<sup>e</sup>More cells affected in midzonal than in centrilobular areas.

**Fig. 1.** Liver sections stained for CYP2E1 and MDM2. Liver sections stained for MDM2 (SMP14) are shown in (A) and (D). In untreated rats there was no nuclear staining for MDM2, but cytoplasmic staining in midzonal areas (A). Serial sections from a rat given DEN 3 h before death is shown in (B) and (C). In the section stained for CYP 2E1 (B), note the presence of positive cells in the central and midzonal areas. The section shown in (C) was first incubated with the CIAP, and subsequently stained for MDM2 with the 2A10 antibody. Note the presence of clearly stained nuclei in the same areas as stained for CYP2E1. CIAP attenuated cytoplasmic staining by the 2A10 antibody. Without CIAP no nuclear staining was seen. (D) A section from a rat given DEN 6 h before death and stained for MDM2. Note the presence of positive nuclei in the central and midzonal areas and the weakened (compare with A) cytoplasmic staining. PT, portal triad; CV, central vein.
central areas was seen. A very weak p53 response in centrilobular and midzonal areas could also be observed (not shown).

Nine hours after injection, nuclear staining for p53 was more distinct and positive nuclei were seen to be distributed equally between midzonal and centrilobular areas. No cytoplasmic staining for MDM2 was seen at this time point. After 12 h, nuclear staining was more pronounced with both antibodies (C and D). A finely granular cytoplasmic staining can also be observed in parts of the midzonal areas depicted in (F). Twenty-four hours following the DEN challenge, the zone containing p53-positive hepatocytes has narrowed considerably (E). The zone staining with the anti-MDM2 antibody was now broader than that with p53-positive cells. Note that in midzonal areas a finely granular cytoplasmic staining can be detected in virtually all of the cells, many of which exhibited only faint nuclear staining (F). No p53-positive nuclei were detected in the corresponding areas (E). PT, portal triad; CV, central vein.

Twelve hours following the DEN challenge, the zone containing p53-positive cells was often more narrow than after 6 and 9 h, but the degree of staining in most of the positive cells was more pronounced (Figure 2C). The intensity of nuclear staining for MDM2 was somewhat increased, with the majority of affected nuclei showing an even, dark staining (Figure 2D). In midzonal areas there was a diffusely spread cytoplasmic staining, which was quite different in character than the cytoplasmic staining observed with untreated rats (Figure 1A).

After 24 h, only the centrilobular zone exhibited positive staining for p53 (Figure 2E). Indeed, virtually all viable hepatocytes of this zone were stained and more heavily stained than...
at previous time-points. In the vicinity of the central vein there were signs of cell death. The zone with nuclei staining for MDM2 was more or less confined to centrilobular areas as well (Figure 2F). In midzonal areas the diffusely spread cytoplasmic staining was further increased. Using the 2A10 antibody, pre-treatment with CIAP did not affect the nuclear staining, but clearly enhanced the cytoplasmic staining in midzonal areas (not shown). Altogether, these changes may reflect phosphorylations and translocations of MDM2, with a translocation from the cytoplasm to the nucleus dominating at earlier time-points, and a transport in the reverse direction in midzonal areas dominating 12–24 h after injection.

Forty-eight hours after treatment the affected zone had narrowed further, and only very few weakly p53-positive cells adjacent to the central vein could be detected after 72 h (not shown). Also, the zone with nuclear MDM2 staining had narrowed (not shown). After 72 h some lobules demonstrated enhanced cytoplasmic staining in midzonal areas, reminiscent of the layer of cells with marked cytoplasmic staining for MDM2 observed in untreated rats (Figure 1A).

Figure 3 shows the number of TUNEL-positive cells. The number peaked 24 h after administration (Figure 3A). Apoptotic cells were localized primarily in the vicinity of the central vein (not shown). The number of hepatocytes exhibiting positive nuclear staining for PCNA, suggesting entry into the S-phase, peaked 24 h following the DEN challenge (Figure 3B). Hepatocytes demonstrating both nuclear and cytoplasmic staining, suggesting entry into the G2-phase, were seen mainly after 48 and 72 h. The time-course of this response is similar to that reported after 2-AAF treatment (34).

Western blot analysis

When immunoprecipitated material was analyzed by western blotting (Figure 4) the time-dependent alterations observed correlated well with those seen immunohistologically. After between 9 and 48 h, two p53 bands were detected [Figure 4A; compare with (35)]. Employing antibodies specific for p53 phosphorylated at Ser-15 and Ser-390 (corresponding to Ser-392/389 of human/mouse p53) gave expected results and suggested that a major portion of the p53 accumulating in nuclei was phosphorylated. Immunoblotting (SMP14) for MDM2 revealed a faint band in untreated rats. The effect of CIAP pre-treatment on the results obtained with the 2A10 antibody is shown in Figure 4C. CIAP markedly increased the band intensity at all time-points, indicating increased levels of phosphorylated MDM2. Band densities reflecting MDM2 levels peaked earlier (9–12 h) than the majority of those reflecting p53 (24 h).

RT–PCR and ISH

Figure 5 depicts mRNA levels for p53, MDM2, p21 and GAPDH. As expected, the level of p53 mRNA was not much altered during the 24 h time-course. On the other hand, the
level of MDM2 mRNA was changed in two phases, with a peak 6 h after treatment, and a more pronounced increase at 24 h (Figure 5A). The 6-h peak preceded the major MDM2 protein increase (at 9 and 12 h; Figures 2 and 4). The increase at 24 h did not correlate to any major change in protein levels, but is consistent with previously published correlations between transcriptional activity and p53 binding to response elements in DNA (36). This and the fact that p21 mRNA was clearly increased at 24 h suggest that the response seen at 24 h was mediated by p53-dependent transactivation. Our MDM2 mRNA analysis does not discriminate between transcripts from two MDM2 promoters, reflecting p53-independent and p53-responsive mRNA accumulation, respectively (11). However, in contrast to transcripts from the human gene, both promoter transcripts from the mouse gene can be induced by DNA-damaging agents and have been shown to support MDM2 protein accumulation (37,38). It is thus possible that the increased MDM2 mRNA levels resulted in increased protein synthesis, but that a rapid turnover of MDM2 prevented increasing protein levels.

ISH of liver slices from untreated rats indicated the presence of low levels of MDM2 mRNA in the periportal/midzonal areas, overlapping with those staining positively for the MDM2 protein. Six and nine hours after treatment this peripheral staining by ISH persisted and occasionally increased, but centrilobular parts remained unchanged and pale. However, after 24 h a predominating centrilobular staining could be seen and now peripheral areas were pale (Figure 6). It seems that the ISH results were consistent with the RT±PCR findings. The data indicate that the 6 h peak (Figure 5) reflected mRNA synthesis in periportal or midzonal areas and that the 24 h increase in mRNA levels reflected synthesis in centrilobular areas.

Studies on pre-neoplastic lesions

Pre-neoplastic lesions can be detected early in the carcinogenic process as GST-P-positive hepatocytes (22). In order to examine the relationship between the expression of MDM2 and GST-P, we re-analyzed liver slices obtained from the rats described in (18). Using MDM2 as a marker for EAF gave the same results as using GST-P as a marker. As documented in Table II, there was a convincing overlap in the staining for GST-P and for MDM2 in sections from all four groups of rats. GST-P-positive EAF (87–95%) also exhibited increased cytoplasmic staining for MDM2. The small differences between the two patterns of staining can be attributed to the fact that the serial sections employed were not identical. Thus, it seems that the cytoplasmic staining for MDM2 is a reliable marker for EAF as staining for GST-P. Furthermore, as p53-positive EAF are the dominating EAF phenotype in these phenobarbital

Fig. 5. Induction of hepatic levels of MDM2 and p21\(^{\text{WAF1/CIP1}}\) mRNA during the 24-h period following treatment of rats with DEN. Liver samples from DEN-treated rats (three rats for each time point except the 1 h time point) were processed as described in the Materials and methods. The levels of MDM2 (A) and p21\(^{\text{WAF1/CIP1}}\), p53 and GAPDH (B) mRNA were measured by comparative RT±PCR analysis at the time-points indicated. The appropriate sized bands for MDM2, p21\(^{\text{WAF1/CIP1}}\), p53 and GAPDH were weighed relative to the band of the internal standard of 18S (324 bp).

*Indicates value significantly different (\(P < 0.05\)) from control value.

(C) RT±PCR products from a single rat. No bands were seen when the reverse transcriptase was omitted from the enzyme mixture (not shown).

Fig. 6. Heterogenous induction of MDM2 mRNA in different hepatic zones 24 h following treatment of rats with DEN. Liver sections were processed for ISH with cRNA probes as described under Materials and methods. Increased expression in centrilobular areas can be seen. Hybridization with the corresponding sense-probe showed no staining (not shown).
treated animals (18), the data indicate that p53 can accumulate in EAF hepatocytes even in the presence of high cytoplasmic levels of MDM2.

In order to assess the lobular distribution of EAF, we examined sections from DEN-treated rats with relatively small EAF (the 2-week group, Table II). Sections stained with the SMP14 antibody exhibit a similar picture as that seen in Figure 2F, and there were small clusters of darker stained cells (Figure 7A), which in serial sections stained for GST-P could be identified as EAF (Figure 7B). Figure 7B shows a typical distribution. The distribution of MDM2-positive EAF within the lobule was assessed. It was found that 57\%\pm12\% of all EAF were localized within the midzonal area with a strong cytoplasmic MDM2 staining and 39\%\pm16\% in the centrilobular area. Very few EAF were found in the periportal area.

Table II. A comparison between GST-P and MDM2 as EAF markers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of EAF/cm² ± SD</th>
<th>Mean EAF area (mm²) ± SD</th>
<th>Area fraction (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GST-P</td>
<td>MDM2</td>
<td>GST-P</td>
</tr>
<tr>
<td>DEN (2 weeks)</td>
<td>10 ± 2</td>
<td>8.7 ± 1</td>
<td>0.016 ± 0.005</td>
</tr>
<tr>
<td>PB (3 months)</td>
<td>9.7 ± 2</td>
<td>8.9 ± 1</td>
<td>0.029 ± 0.008</td>
</tr>
<tr>
<td>DEN (10–12 weeks)</td>
<td>101 ± 2</td>
<td>96 ± 32</td>
<td>0.27 ± 2</td>
</tr>
<tr>
<td>PB (14 months)</td>
<td>43 ± 19</td>
<td>37 ± 9</td>
<td>0.38 ± 0.2</td>
</tr>
</tbody>
</table>

*Three rats were given an initiating dose of DEN at birth and two or 10–12 weekly doses of DEN after weaning.

**Three rats were given an initiating dose of DEN at birth and after weaning phenobarbital (500 p.p.m. in drinking water).

*Liver sections from two rats were analyzed.

We also analyzed rats treated for a longer time but with very low doses (10 weekly doses; 0.025 mmol/kg) of DEN. The number of GST-P-positive EAF/cm², the mean EAF area and EAF area fraction are presented in Table III. Using MDM2 as a marker gave similar results as using GST-P (not shown). These rats had smaller lesions than those presented in Table II, and their localization in the lobule could easily be assessed, especially when using MDM2 as a marker. It was found that 84\% of these small lesions were localized in the area with strong cytoplasmic staining for MDM2 (Table III). Sixteen percent were found in the centrilobular area. No lesions were found in the periportal area.

**Discussion**

In the present study we describe two different types of p53/MDM2 responses in rat liver to a challenge by DEN (most data...
data indicate that this peak reflected midzonal and perhaps also latory feedback loop. Further support for the existence of such of p53/MDM2 protein in centrilobular areas observed here are accompanied by limited cell death. The changes in the levels of p53/MDM2 protein were analyzed in sections stained for GST-P and localization of EAF was analyzed in serial sections stained for MDM2.

Table III. Zonal localization of GST-P/MDM2-positive EAF in DEN-treated rats

<table>
<thead>
<tr>
<th>EAF parameters</th>
<th>No. EAF/cm² ± SD</th>
<th>Mean EAF area ± SD</th>
<th>Area fraction% ± SD (ar)</th>
<th>Localization of EAF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrilobular area</td>
<td>16 ± 24</td>
<td>84 ± 24</td>
<td>0.023 ± 0.014</td>
<td>16 ± 24</td>
</tr>
<tr>
<td>Midzonal area</td>
<td>0.0034 ± 0.0013</td>
<td></td>
<td>0.023 ± 0.014</td>
<td>0.023 ± 0.014</td>
</tr>
<tr>
<td>Periporal area</td>
<td>0.0013 ± 0.014</td>
<td></td>
<td>0.0034 ± 0.0013</td>
<td>0.0034 ± 0.0013</td>
</tr>
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</table>

Liver sections from three rats were analyzed. Rats were given 10 weekly doses of DEN (0.025 mmol/kg) intraperitoneally. Prior to death the rats received a challenging dose of 0.99 mmol DEN/kg. EAF parameters were analyzed in sections stained for GST-P and localization of EAF was analyzed in serial sections stained for MDM2.

The 6 h mRNA peak correlated with small changes in p53 protein levels, and other factors might have induced it. Possible candidates are IGF or FGF (40,41) and ATM may provide a direct link to DNA damage (7–9). This kinase has been implicated previously in the response to DEN (19) and other alkylating agents (42). More recent data indicate that ATM/c-Abl phosphorylates MDM2 at a CIAP accessible site (Tyr394). This may occur in the nucleus and lead to an accumulation of p53 (43,44). It is thus possible that the nuclear phosphorylation seen in Figure 1C contributed to the accumulation of p53.

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We find that EAF of all sizes, and in all animals examined, express high levels of cytoplasmic MDM2. Thus, even though our database is limited we have indications that MDM2 might be used as a marker for EAF. The stability of the marker in more advanced lesions remains to be studied, as well as its reliability in other protocols. It can also be concluded that EAF hepatocytes exhibit a MDM2 phenotype similar to that in many unaltered hepatocytes in midzonal areas. We also find that small EAF induced by repeated low doses of DEN preferentially localize to the same midzonal areas. These relationships indicate a governing role for MDM2 expression in EAF initiation and/or early development. It has been indicated that EAF hepatocytes are the progeny of unaltered hepatocytes (45), but the exact role of MDM2 in EAF development, and if it for example affects GST-P expression, remains to be characterized.

By comparing earlier published data (18) and data presented here (Table II), it is indicated that only ~60% of EAF with the MDM2-positive phenotype exhibited a p53-negative phenotype after 2 weeks treatment with DEN. After 10–12 weeks treatment the corresponding figure was close to 100%. This suggests a slower development of the p53-negative phenotype than of the MDM2-positive phenotype. Our data also indicate that p53 readily accumulated in cells with a high MDM2 expression in the cytoplasm. This was seen in a few EAF hepatocytes in DEN-treated rats and in most EAF hepatocytes in phenobarbital-treated rats. The data indicate that there is no direct coupling between the MDM2 phenotype and the attenuated p53 response.

In summary, we present data reflecting two types of p53/MDM2 responses, one in centrilobular areas and one in midzonal areas. The sequence of the p53 and MDM2 responses in centrilobular areas is compatible with the existence of an operative feedback loop. The short response in midzonal areas cannot be explained on the basis of toxicokinetic differences. Instead, we find a high constitutive expression of MDM2 and a more rapid MDM2 mRNA accumulation in this area. Our data indicate a role for the midzonal type of MDM2 response in early EAF development: EAF hepatocytes exhibited...
a MDM2 phenotype similar to that seen in midzonal cells and the distribution of these two cell types was overlapping. Considered as a whole, our data suggest that the DEN-induced initiation process is not stochastic among hepatocytes, but support the hypothesis that many EAF are initiated via epigenetic alterations.

References


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