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Lack of p53 protein expression in preneoplastic rat hepatocytes *in vitro* after exposure to N-acetoxy-acetylaminofluorene, X-rays or a proteasome inhibitor

H.E. van Gijssel [a], U. Stenius [b], G.J. Mulder [a], J.H.N. Meerman [a,*]

[a] *Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands*
[b] *Institute for Environmental Medicine, Karolinska Institute, Stockholm, Sweden*

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Abstract

Clonal expansion of initiated cells is an important process in carcinogenesis. Loss of functional p53 protein in initiated, preneoplastic cells might be involved in this process because such a loss would favour cell growth at the expense of normal cells upon exposure to genotoxic compounds. We have tested the hypothesis that p53 is not expressed in preneoplastic cells in the rat liver. Hepatocytes were isolated from livers of 10-week-old female rats that contained foci of preneoplastic hepatocytes, generated by 6–7 weekly injections of diethylnitrosamine (0.15 mmol/kg body wt intraperitoneally (i.p.)), starting 24 h after birth. The mixture of phenotypically normal and preneoplastic hepatocytes was exposed to X-rays or N-acetoxy-acetylaminofluorene (NAAAF), both causing DNA damage directly. At 24 and 48 h after exposure the cells were fixed and double stained for glutathione-S-transferase 7-7 (GST7-7), to identify preneoplastic cells, and p53. The percentage of p53-positive cells was much lower in GST7-7 positive (GST7-7$^+$) than in GST7-7 negative (GST7-7$^-$) hepatocytes. Exposure of cells to X-rays or NAAAF induced p53 in GST7-7$^-$ cells after 24 h, but GST7-7$^+$ hepatocytes failed to do so. These results suggest that preneoplastic cells do not express p53 or have an attenuated p53 response to genotoxic treatments. This was confirmed when the cells were exposed to a proteasome inhibitor, PSI, which inhibits p53 degradation: a 12-fold increase in p53-positive cells was found after 48 h in GST7-7$^-$ hepatocytes, but in GST7-7$^+$ hepatocytes no increase was observed. The percentage of GST7-7$^+$ hepatocytes among surviving cells was increased after exposure to NAAAF, suggesting that these are more resistant to NAAAF than GST7-7$^-$ cells. This was not observed with PSI. These results indicate that preneoplastic hepatocytes have a lower p53 protein content and are not able to increase p53 upon inhibition of p53 breakdown or upon induction of DNA damage. Therefore, loss of p53 may favour clonal expansion of preneoplastic hepatocytes in the rat after administration of hepatocarcinogens or X-rays.

Keywords: Preneoplastic heptatocytes; p53; DNA damage; Clonal expansion; X-rays; Carcinogenesis

1. Introduction

Chemical carcinogenesis is a multistage process in which induction of DNA damage and mutations leading to genetically and phenotypically altered cells is essential. Such cells emerge early in the process of carcinogenesis, for instance in human colon, breast and stomach. They can be induced experimentally, e.g. in rat liver *in vivo* by administration of chemical carcinogens [1] and are recognisable by the expression of markers like glutathione-S-transferase 7-7 (GST7-7) and γ-glutamyltranspeptidase. They are often referred to as preneoplastic or enzyme-altered (EA) cells. Their number and subsequent rate of expansion are related to the potency of the initiating and promoting compound, respectively [2]. It has been suggested that the expansion of EA cells is clonal in nature [3]. The mechanisms that ensure the selection and clonal expansion of the EA cells are complex. Different mechanisms may be responsible for clonal expansion: e.g. selective growth stimulation of preneoplastic cells, selective inhibition of the growth of normal cells, and a decreased rate of apoptosis in

* Corresponding author at Division of Molecular Toxicology, LACDR, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV, Amsterdam, The Netherlands.
E-mail address: meerman@chem.vu.nl (J.H.N. Meerman).

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preneoplastic cells. All will provide a growth advantage to preneoplastic cells.

The tumour suppressor gene *TP53* plays an important role in the process of carcinogenesis. One of its physiological roles is to block the cell cycle in the G1-phase after DNA damage, allowing the cell to repair its damaged DNA or to trigger apoptosis (reviewed in [4, 5]. Levels of the p53 protein are increased in many cell types after various types of DNA damage [6–8] due to stabilisation of the protein by phosphorylation and/or acetylation [4, 9]. We have shown that genotoxic compounds with promotional activity in the rat liver are able to induce p53 in normal rat liver resulting in growth retardation in normal cells [10], and have suggested that this may allow clonal expansion of EA cells on the premise that these cells have lost the capability of expressing functional p53. Stenius and colleagues showed that EA-hepatocytes in primary culture and *in vivo* did not respond to diethylnitrosamine (DEN) exposure by induction of p53 [11, 12]. Such a lack of p53 expression in EA cells might be due to a loss of intrinsic capacity to increase p53 levels in response to DNA damage or to overexpression of proteins that promote the breakdown of p53, such as Mdm2 [13, 14]. Alternatively, a decreased capacity to metabolise DEN to its reactive, genotoxic metabolite (due to the altered expression of biotransformation enzymes in EA hepatocytes) [15, 16] might also be the cause of this lack of effect of DEN.

In order to avoid the possibility of a decreased bioactivation, we have subjected hepatocytes isolated from EA foci-bearing rats (a mixture of GST7-7$^-$ and GST7-7$^+$ cells) *in vitro* to treatments that cause DNA damage directly; these included N-acetoxy-acetylaminofluorene (NAAAF), a metabolite of the hepatocarcinogen 2-acetylaminofluorene (2-AAF) that does not require further bioactivation and X-irradiation that produces DNA damage directly. We also used proteasome inhibitor I (PSI) that inhibits the degradation of p53 [17].

2. Materials and methods

2.1. Antibodies and chemicals

DEN was purchased from Sigma (St Louis, MO, USA). PAb 240 was from Santa Cruz (Santa Cruz, California, USA). Rabbit polyclonal anti-glutathione-S-transferase π antibody was raised against human GSTp-protein (the latter was a kind gift from D.J. Meyer, Middlesex School of Medicine, London, UK). This antibody cross-reacts with rat GST7-7. Goat anti-mouse antibody, swine anti-rabbit antibody, alkaline phosphatase anti-alkaline phosphatase (APAAP)-mouse monoclonal antibody, peroxidase anti-peroxidase (PAP)-rabbit polyclonal antibody and New Fuchsin substrate system were from DAKO, Denmark. NAAAF was synthesised according to Beland and associates [18]. PSI was from Calbiochem-Novabiochem Corporation (San Diego, CA, USA).

2.2. Donor animals, cell culture and treatment

Pregnant Sprague–Dawley rats were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) or B&K Universal Ltd (Stockholm, Sweden). Newborn female rats were injected intraperitoneally (i.p.) within 24 h after birth with 0.15 mmol/kg DEN diluted in saline. After 3 weeks the rats were weaned and injected with 0.15 mmol/kg DEN i.p. once a week for 6 weeks. Each rat received 6 or 7 injections of DEN. Cells were isolated one week after the last injection and cultured in RPMI 1640 medium as previously described [19]. The cells were seeded on collagen-coated dishes at a density of 2.5×10^5 cells/35 mm dish and were left to attach for 1.5 h. After the medium was changed, NAAAF dissolved in dimethylsulphoxide (DMSO) was added to the medium (final concentration 0.5% (v/v) DMSO). PSI was diluted in medium and added to the dishes after the medium had been changed. Cells were left untouched for 24 h. Cells were X-irradiated in medium using an Andrex Smart 225 X-ray machine (200 kV and 4.0 mA; Andrex, Copenhagen, Denmark) delivering a dose of 1.24 Gy/min. The dose was measured with a PTW-dosimeter with a 0.02 cm^3 ionising chamber (PTW, Freiburg, Germany). The medium was changed directly after irradiation. Medium was changed 24 h after all treatments.

Cells were fixed in 4% (v/v) formaldehyde in phosphate buffered saline (65 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) at 24 and 48 h after treatment and stained by immunohistochemistry.

2.3. Immunohistochemistry and evaluation.

Double staining for p53 and GST7-7 was performed as previously described [11]. The percentage of positively stained cells was determined by counting at least 500 GST7-7$^-$ and 200 GST7-7$^+$ cells in randomly selected fields. The code of the slides was blinded for the evaluator. The number of attached cells was determined after 24 and 48 h by counting ten fields per dish at random. This represents the number of surviving cells because dead cells were detached. All experiments were repeated at least three times. Data were analysed statistically with the Student *t*-test.

3. Results

Approximately 5–10% of the isolated hepatocytes were positively stained for GST7-7 in their cytoplasm,

indicating their preneoplastic nature. The p53 protein could be detected in the nuclei of some GST7-7$^-$ cells before exposure *in vitro*: the percentage of p53-positive cells varied between 4 and 8% at 24 h and 0.4–3% at 48 h after treatment (Fig. 1). Hardly any p53-positive cells were found among GST7-7$^+$ cells before exposure *in vitro* (0–1%) (Fig. 1).

Increased percentages of p53-positive cells were found in GST7-7$^-$ hepatocytes after exposure to NAAAF, X-rays and PSI (Fig. 1). The highest levels were found after exposure to as little as 0.5 μM NAAAF (Fig. 1a). X-irradiation of cells increased the percentage p53-positive cells in GST7-7$^-$ cells maximally 1.6-fold compared with control levels after 24 h (Fig. 1a); however, no increased levels of p53-positive cells were found 48 h after X-irradiation (Fig. 1b). The percentage of p53-positive cells was lower 48 h after NAAAF or X-ray exposure compared with levels 24 h after exposure. PSI did not induce p53 levels after 24 h (data not shown), but a pronounced 12-fold increase was observed after 48 h (Fig. 1b).

In GST7-7$^+$ hepatocytes only 1.1% ± 0.3% of the cells were positively stained for p53 at the highest dose of NAAAF used (Fig. 1a). No p53-positive GST7-7$^+$ cells were found after exposure to lower concentrations of NAAAF (Fig. 1a) or 48 h after exposure (Fig. 1b). Neither was an increase of p53-positive cells found in GST7-7$^+$ cells after X-ray or PSI exposure (Fig. 1a and b).

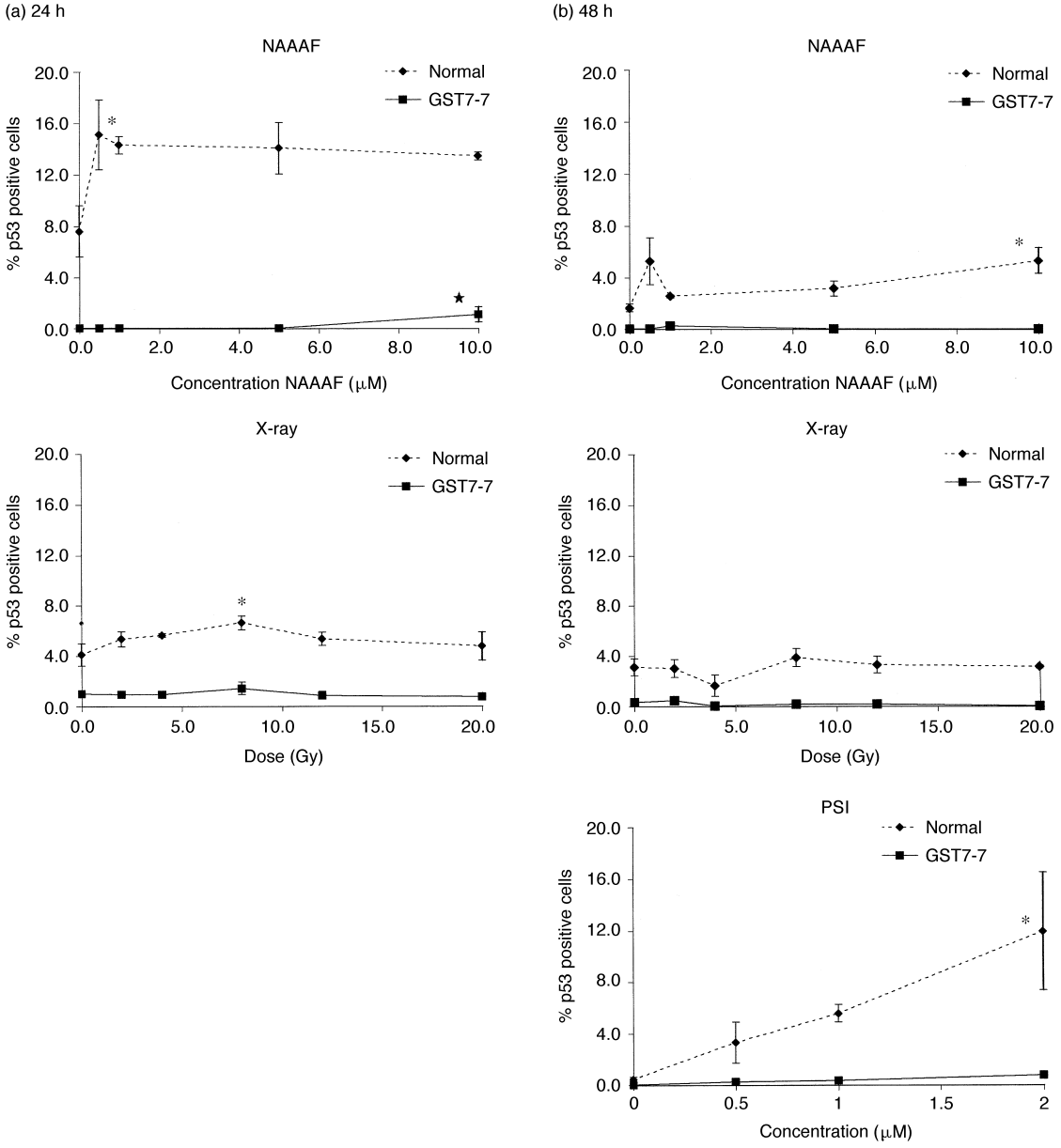

Fig. 1. The effects of NAAAF, X-rays, and PSI on the percentage of p53-positive cells in normal GST7-7$^-$ (-◆-) and GST7-7$^+$ (-■-) hepatocytes *in vitro* after 24 h (a) or 48 h (b). *Indicates significantly different from untreated cells at $P < 0.05$ (Student *t*-test).

Cell survival after 24 h decreased at higher concentrations of NAAAF and doses of X-rays; a further decrease was observed after 48 h. NAAAF exposure led to the highest percentage of cell death: only 26% of the cells survived at the highest concentration after 48 h (Fig. 2b). Much less cell death was found after X-irradiation: after 48 h, 68% of cells still survived (Fig. 2b). PSI also affected the survival of cells: only 32% of cells had survived after 48 h (Fig. 2b).

The percentage of GST7-7$^+$ cells among the surviving cells slightly increased during 48 h under control conditions which is due to the culture conditions, as previously reported [20], but a pronounced increase was found 24 and 48 h after 10 μM NAAAF exposure: from $5.0 \pm 1.0\%$ to $14.8 \pm 2.2\%$ and $11.8 \pm 3.2\%$ to 20.6 ± 2.5, respectively (Fig. 2a and b). This suggests that GST7-7$^+$ cells are more resistant to exposure to NAAAF than GST7-7$^-$ hepatocytes. In addition, the percentage of GST7-7$^+$ cells 48 h after X-irradiation with 8 Gy tended to be higher (Fig. 2b); however, this was not statistically significant. The exposure to PSI did not increase the percentage of GST7-7$^+$ cells at 48 h (Fig. 2b).

4. Discussion

The data presented in this paper show that exposure *in vitro* to NAAAF, X-rays or PSI resulted in an increased percentage of p53-positive nuclei in GST7-7$^-$

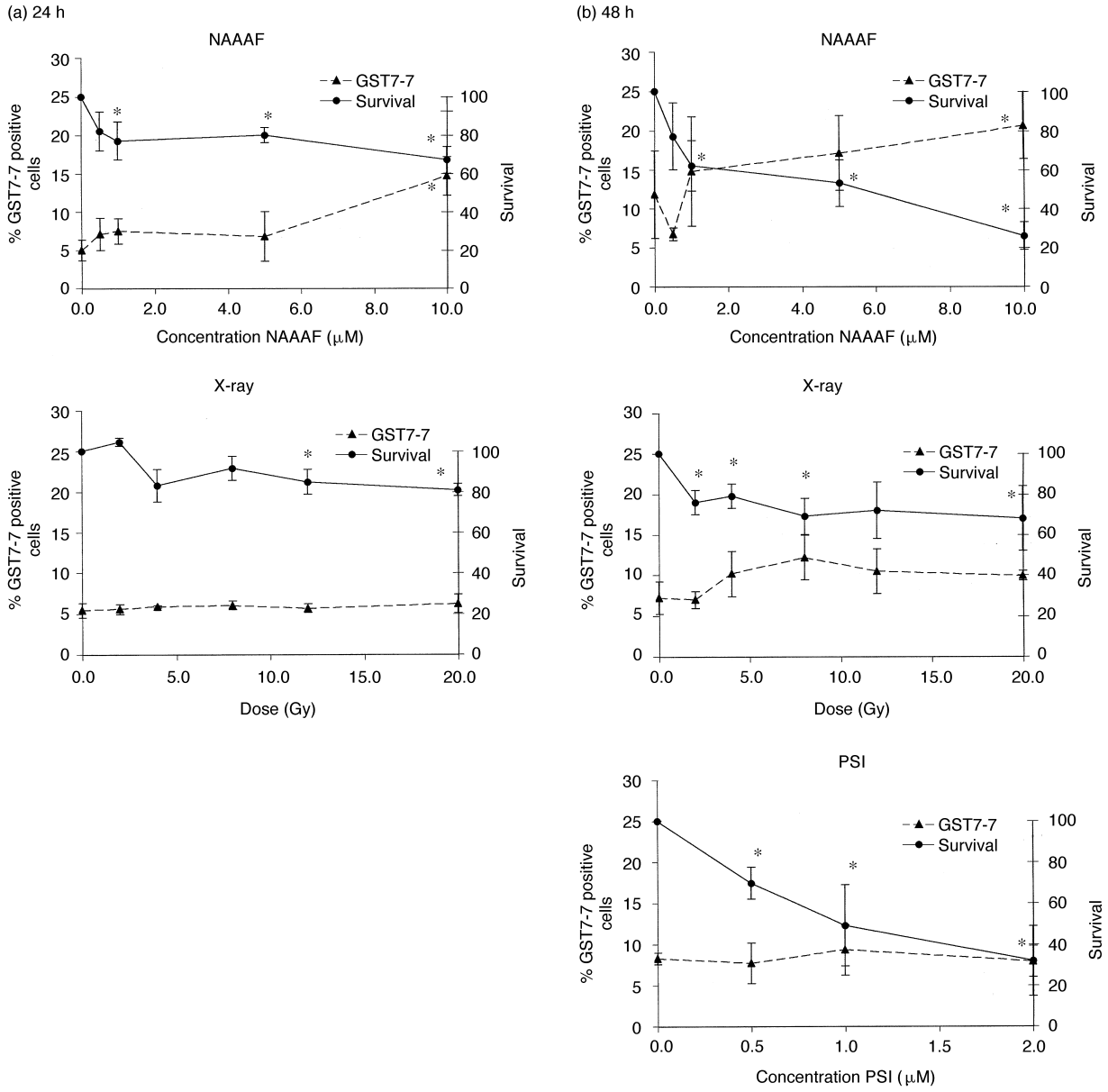

Fig. 2. The effects of NAAAF, X-rays, and PSI on the percentage of GST7-7$^+$ cells among the total surviving cells (-▲-) and survival of total hepatocytes *in vitro* (-●-) 24 h (a) or 48 h (b) after exposure. *Indicates significantly different from untreated cells at $P < 0.05$ (Student *t*-test).

hepatocytes isolated from EA foci-bearing rats. In contrast, GST7-7$^+$ hepatocytes isolated from the same rats, hardly express p53 *in vitro* in the control situation and show no increase after exposure to NAAAF, X-rays or PSI.

In this *in vitro* study p53 staining was found in some control GST7-7$^-$ hepatocytes, whereas no staining was found *in vivo* in control animals [10, 21, 22]. This might be a reaction to the stress induced by the cell isolation procedure. It is unlikely that the increased p53 level in these hepatocytes is solely caused by persistent DNA damage induced by the DEN treatment used to generate the EA foci [23] because such damage should also have induced p53 *in vivo* (which was not the case). However after isolation, hepatocytes enter the cell cycle and synthesise DNA as indicated by their incorporation of [^{3}H]-thymidine or bromo-deoxyuridine [11, 19]. The p53 protein is typically expressed during the G1 phase of the cell cycle [10, 24] and the percentage of cells with high p53 after genotoxic damage is proportional to the percentage of cells in G1 [25]. Therefore, the higher expression in control GST7-7$^-$ cells *in vitro* may be due to cells having entered the G1 phase after isolation. In contrast, control GST7-7$^+$ hepatocytes *in vitro*, hardly expressed p53 (Fig. 1), indicating that GST7-7$^+$ cells either have intrinsically lower levels of p53 than GST7-7$^-$ cells or do not as readily as GST7-7$^-$ cells enter the G1 phase *in vitro*. However, the latter possibility can be excluded because it has been shown that GST7-7$^+$ and GST7-7$^-$ cells *in vitro* have similar basal rates of proliferation as shown by their equal [^{3}H]thymidine incorporation rates [11].

PSI does not induce DNA damage but blocks p53 degradation [17]. Thus, PSI is expected to increase p53 levels because, under physiological circumstances, p53 levels remain low due to rapid degradation of p53 by the ubiquitin–proteasome pathway [26]. The fact that GST7-7$^+$ cells, contrary to GST7-7$^-$ cells, show no increased p53 level after PSI exposure, may indicate that p53 is expressed at a much lower rate in these cells. Furthermore, this may explain why these cells are not able to respond to DNA damage by inducing p53.

p53 was induced in the nuclei of an increased percentage of GST7-7$^-$ cells after DNA damage caused by X-irradiation or NAAAF. The nuclear localisation of p53 after the various treatments agrees with earlier publications [6–8, 21]. NAAAF increased the percentage of p53-positive cells in the GST7-7$^-$ cell population *in vitro* 2- to 3-fold. The 1.6-fold increase by X-ray is comparable with the effect observed in normal hepatocytes *in vivo* in rats and mice [22, 27]. PSI increased p53 levels 12-fold in GST7-7$^-$ cells, which agrees with its effect in RAT-1 and PC-12 cells [17]. The levels of p53 were higher 24 h after NAAAF treatment than after 48 h. This might be due to repair of DNA damage followed by the loss of p53. Interestingly, at most 15% of cells were positive for p53 after NAAAF exposure. This may be due to the fact that not all cells are in the same phase of the cell cycle at the same time after isolation [11, 19] (see also discussion above).

Stenius and colleagues showed that p53 is induced in hepatocytes *in vivo* and *in vitro* after DEN exposure and that the percentage of p53-positive cells is lower in GST7-7$^+$ cells [11, 12]. The latter could partially be due to lower metabolic activation of DEN in GST7-7$^+$ cells. However, metabolic activation does not play a role during exposure to NAAAF and X-rays because they damage DNA directly. Therefore, our results suggest that GST7-7$^+$ cells are not able to induce p53 after DNA damage.

GST7-7$^+$ hepatocytes have an altered enzyme expression and higher levels of glutathione that might influence the detoxification rate of NAAAF. However, reactive intermediates derived from reactive esters of N-OH-AAF (such as NAAAF) that may react with DNA do not readily react with glutathione [28]. Indeed, depletion of glutathione in rats *in vivo* did not change the covalent binding of N-OH-AAF to DNA, although formation of AAF–glutathione conjugates had decreased [29]. As yet, we cannot definitely exclude the possibility that some form of (enzymatic) detoxification is higher in GST7-7$^+$ than in GST7-7$^-$ hepatocytes. However, this does not play a role with X-ray-induced DNA damage because X-rays induce DNA damage directly [30]. Cells cannot prevent this damage and, therefore, altered enzyme expression cannot affect this type of DNA damage. Thus, we conclude that GST7-7$^+$ hepatocytes are not able to induce p53 after DNA damage.

The percentage of GST7-7$^+$ cells among the surviving cells was increased 48 h after exposure to NAAAF. Since p53 may induce apoptosis, GST7-7$^+$ hepatocytes may be more resistant to the induction of apoptosis than GST7-7$^-$ hepatocytes. This seems to contrast with data from several other groups that show that the rate of apoptosis in preneoplastic hepatocytes *in vivo* is higher than in normal cells [31–34]. However, these groups also showed that apoptosis in preneoplastic cells is greatly reduced by various tumour promoters. As the preneoplastic, GST7-7$^+$ cells in this study were isolated shortly after the administration of DEN, which acts as a strong promoter, a similar reduction in the incidence of apoptosis may have occurred in these cells.

The relative increase of GST7-7$^+$ cells *in vitro* is consistent with the hypothesis that a selection for GST7-7$^+$ cells may occur after induction of DNA damage in the liver *in vivo*. It also agrees with data showing that GST7-7$^+$ hepatocytes are able to proliferate under conditions where proliferation of GST7-7$^-$ cells is blocked upon DNA damage [11, 35].

In conclusion, our results show that GST7-7$^-$ cells respond to DNA damage with p53 induction and that

levels of p53 increase after inhibition of p53 degradation. In contrast, GST7-7$^+$ cells do not respond with p53 induction to these events. GST7-7$^+$ cells, therefore, may proliferate when exposed to carcinogens because their cell cycle is not blocked. This may result in their clonal expansion. Additionally, it may lead to the accumulation of mutations in these cells. Griffith and colleagues already showed that the absence of p53 permits outgrowth of mutated cells after DNA damage [36]. Thus, a mechanism of clonal expansion of GST7-7$^+$ cells after induction of DNA damage may, indirectly, be based on selective inhibition of proliferation of GST7-7$^-$ cells due to increased p53 levels in the latter cells. Further research has to be done to reveal the mechanism(s) that prevent(s) the expression of p53 in GST7-7$^+$ cells.

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