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Cell cycle arrest and modulation of HO-1 expression induced by acetyl salicylic acid in hepatocarcinogenesis

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Abstract

Background and aims: Control of cell proliferation is important for cancer prevention since cell proliferation has an essential role in carcinogenesis. In rodent carcinogenesis models, antioxidant agents suppress carcinogen-induced cellular hyper proliferation in the target organs. Strict control of cell division is an essential process to ensure that DNA synthesis and mitotic division are accurately and coordinately executed. We studied the interplay between cell cycle and heme oxygenase-1 (HO-1) and the effect of the acetylsalicylic acid (ASA) in hepatic carcinogenesis. *Methods:* Male *CF1* mice pre-treated with dietary *p*-dimethylaminoazobenzene (DAB; 0.5%, w/w) were fed with ASA (0.16%, w/w). We investigated the hepatic expression of cyclin D1, cyclin E, Cdk2, Cdk4, p21, p27, p53; the level of bcl-2, an antiapoptotic protein and of heme oxygenase-1 (HO-1), a marker of oxidative stress, by Western blot analysis. *Results:* The treatment with ASA produced an important attenuation in the induction of cyclin E and cyclin D1 provoked by DAB. p21 and p27 levels were increased when animals received both drugs. The administration of ASA to DAB treated animals induced Cdk2 (29%). HO-1 induction (65%) provoked by DAB was diminished by ASA administration reaching lower induction levels (23%). *Conclusion:* The deregulation of cyclin/CDK expression and the up-regulation of p21 and p27 with the administration of ASA, post-treatment of the carcinogen administration, would block the pass through out to the G0/G1 check point to permit the cells to repair their DNA and HO-1 protected the liver from reactive oxygen species produced from DAB.

Keywords: Acetyl salicylic acid (ASA); Cell cycle; Heme oxygenase-1 (HO-1); Oxidative stress; Hepatocarcinogenesis

1. Introduction

The liver is one of the most common target organs of xenobiotic insult in chronic toxicity and carcinogenicity studies. Molecular alterations at the subcellular and genetic levels will become of importance to evaluate the carcinogenic mechanism in experimental animals and will be of relevance to risk assessment in human populations. Tumorigenesis is a multistep process in which mutation in genes involved in cell cycle control and apoptosis accumulate over time (Senderowicz, 2001).

A proliferation stimulus leads to activation of a death signal, either directly as a consequence of entry into the cell cycle or through activation of a parallel pathway, therefore successful proliferation can only occur if the apoptotic program is suppressed.

Cyclins are essential components of the cell cycle machinery. They function to bind and activate their

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specific cyclin dependent kinase (CDK) partners. During progression through the G_1 phase of the cell cycle, two major types of cyclins are required: D-type cyclins and cyclin E (Nurse, 2000).

The major function of the cyclin D pathway is to provide a link between mitogenic stimulus and the potentially autonomous cell cycle machine. Conversely, constitutive activation of the cyclin D pathway can reduce or overcome certain mitogen requirements for cell proliferation and thereby contribute to oncogenic transformation (Sherr & Roberts, 1999). Cell cyclin response to either mitogen deprivation or genotoxic stress requires CDK inhibitors (CKIs) of the CIP/KIP family, which includes $p21^{cip1}$ and $p27^{kip1}$ (Morgan, 1995). Increased protein levels of D cyclins result in complex formation with their CDK partners, which function to sequester $p21^{cip1}$ and $p27^{kip1}$ away from cyclin E-CDK2 complexes, allowing G1-S progression (Perez-Roger, Kim, Griffiths, Sewing, & Land, 1999).

Reactive oxygen species (ROS) are critically involved in multistage carcinogenesis (Klaunig et al., 1998). Heme oxygenase (HO) is a heme-catabolising enzyme that converts heme into biliverdin, iron and carbon monoxide. HO-1, the inducible form of HO, is thought to act as an endogenous antioxidant defense mechanism (Maines, 1997). Recent attention has been focused on the biological effects of the product(s) of this enzymatic reaction that have important antioxidant, anti-inflammatory and cytoprotective functions (Elbirt & Bonkovsky, 1999). Cellular over expression of HO-1 up-regulates p21, diminishes proliferative cell growth, and confers marked resistance to apoptosis. Consequently up-regulation of p21 contributes to the altered pattern of cell growth and resistance to apoptosis. The inductive effect of HO-1 on p21 and the inhibitory effect on the cell cycle provide a hitherto unsuspected mechanism underlying the cytoprotective action of HO-1 (Inguaggiato et al., 2001). While the cellular processes leading to HO-1 induction are complex and tightly regulated, one common feature to most of the stimuli that up-regulate HO-1 is a significant shift in cellular redox. The induction of HO-1 mRNA expression during the hepatocarcinogenesis chemically induced with *p*-dimethylaminoazobenzene (DAB) in mice, in response to these stimuli, was proposed to have a protective role (Vazquez et al., 2002). Hill-Kapturezak et al. (2000) suggested that the induction of HO-1 mRNA in human kidney requires the novo transcription and is an adaptative response to the inflammatory effects of TGF-β1.

It has been shown that aspirin inhibits ROS-mediated DNA damage (Hsu & Li, 2002). Regular use of aspirin and other non-steroidal anti inflammatory drugs has been consistently associated with reduced risk of colorectal cancer and adenoma (Pollet et al., 1999) and there is some evidence for a protective effect for other types of cancer (Thun, Honley, & Patrono, 2002).

In this study, we have examined the expression of cell cycle regulatory proteins (cyclins and CDKs) as well as inhibitors of cell cycle (p21, p53 and p27) in mouse liver. Because of the known oxidative stress provoked by the carcinogen, we have also investigated the expression of HO-1, considered a key cellular defense mechanism and an inflammatory mediator, and we have evaluated if the expression of HO-1 and cell cycle regulators was differentially affected by the treatment with the anti inflammatory drug ASA, in this in vivo model of liver cancer.

Our aim is to study the power of this drug to revert the DNA damage and to know the status of the proteins of the cell cycle checkpoints that represent integral components of DNA repair, which coordinate the actions of the cell cycle machinery and the HO biochemical pathway acting together as a protective response to the damage and restoration of the DNA structure.

2. Materials and methods

2.1. Chemicals

Chemicals were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals and treatment

Male *CF1* mice (30 g) ($n = 24$) received a standard laboratory diet (SLD), Purina 3 (Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires) supplemented or not with *p*-dimethylaminoazobenzene (DAB; 0.5%, w/w) and/or acetylsalicylic acid (ASA; 0.16%, w/w) under the intoxication protocol shown in Fig. 1. ASA dose was selected according to the dose previously used for chemoprevention of lung tumori-

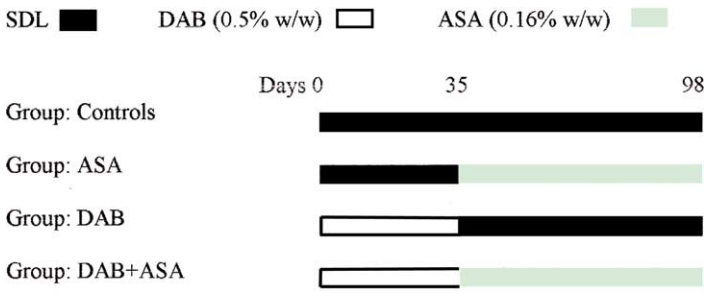

Fig. 1. Experimental schedule.

genesis (Duperron & Castonguay, 1997). A group (DAB) of animals ($n = 6$) were placed on dietary DAB during 35 days and fed with SLD for an additional period of 63 days. Control animals ($n = 6$) received SLD for a whole period of 98 days. Two other groups non-treated (ASA) and DAB treated (DAB+ASA) animals ($n = 6$ each) received ASA in the diet since day 36. All animals received food and water ad libitum.

All animals were inspected at least twice daily. Body weight and food consumption were measured at intervals throughout the study. Food was removed for animals 16 h before sacrifice. Mice were killed under ether anaesthesia at the indicated times and liver samples were immediately processed. Animals received human care and were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC) and in accordance with the UK Guidelines for the Welfare of Animals in Experimental Neoplasia (UK Coordinating Committee on Cancer Research, London, 1998).

2.3. Protein extraction and Western blotting

Expression of cyclin D1, cyclin E, Cdk2, Cdk4, p21, p27, p53, bcl-2 and HO-1 proteins was studied by Western blot analysis. Protein extraction from liver tissues (100 mg chopped in small pieces) was obtained by using lysis buffer (50 mM Tris–HCl, pH 6.8; 10% SDS) and homogenised. After 30 min of incubation at 4 °C, the lysates were heated at 100 °C during 5 min and were centrifuged at 10,000 × g for 30 min at 4 °C. Lysates containing equal amounts of proteins (100 μg) were resolved on 7.5–12.5% SDS–PAGE (the percentage depending on the molecular weight of the proteins to be detected). Rainbow coloured protein molecular weight standards obtained from Amersham-Pharmacia Biotech were used for the estimation of molecular size. The proteins were blotted to a Hybond-ECL nitrocellulose membrane that was probed and washed according to the instructions for the enhanced chemiluminescence Western blotting detection system (Amersham-Pharmacia Biotech), with transfer buffer (pH 8.3) containing 20% (v/v) methanol using a Hoefer miniEV electrotransfer unit (Amersham-Pharmacia Biotech). The membrane with transferred proteins was blocked with 5% dry unfat milk in TBS 1× containing 0.1% Tween 20 (TBST) for 1 h at room temperature, and incubated with the first antibody diluted in TBST for 1 h at room temperature. After washing in TBST, the membrane was incubated with horseradish peroxidase-labelled secondary antibody for 1 h at room temperature.

The following first polyclonal antibodies were used: goat p21 (C19)-G, rabbit p53 (FL393), goat Cdk2 (M2)-G, goat Cdk4 (H-22)-G, rabbit cyclin D1 (H-295), rabbit cyclin E (M20), goat bcl-2 (N-19)-G (Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit p27 (Ab-1) (Oncogene) and rabbit anti-heme-oxygenase-1 (StressGen). Goat actin (I-19) (Santa Cruz Biotech) was used as a loading control. The second antibodies were anti-goat IgGHRP or anti-rabbit IgGHRP (Santa Cruz Biotech). For quantification of immunoblots, relative intensity of bands was quantified by densitometry using ImageMaster image analysis software (Amersham-Pharmacia Biotech). Control for loading and transfer was obtained by probing with anti-β-actin. The values of the different proteins bands were normalised for the intensity of the loading control.

2.4. DNA fragmentation assay

Samples of tissue liver of animals intoxicated with DAB were incubated at 37 °C with lysis buffer (50 mM Tris–HCl, pH 8.0; 20 mM EDTA; 10 mM NaCl; 1.0% SDS) and pancreatic RNAse for 15 min with mix by flicking the tube. Then, Proteinase K solution was added at a final concentration of 100 μg/ml and incubated for 55 °C until the tissue disappeared completely or it was mostly lysed, mixing occasionally during the incubation to disperse the sample. The DNA was extracted twice with equal volumes of phenol and once with chloroform. The DNA was then precipitated with 1 ml of sodium acetate 2.0 M (pH 4.8) and 2.5 ml of ethanol at −20 °C overnight and centrifuged at 13,000 × *g* for 30 min. Samples were electrophoresed in 1.4% agarose gel and DNA was visualised by ethidium bromide staining.

2.5. Statistical analysis

Statistical analysis was performed by Student's *t*-test. Data are presented as means ± S.D. and significance was defined as $P < 0.05$.

3. Results

3.1. Cell cycle related proteins and Bcl-2 in chemically induced hepatocarcinogenesis

Progression through the cell cycle is driven by the periodic activation of several CDKs. CDKs activity is partially regulated by CDK inhibitors. The expression of cyclin D1, cyclin E, their partners Cdk4 and Cdk2, the cell cycle inhibitors p21, p27 and p53 and the antiapoptotic protein bcl-2 was analysed by Western blot and is shown in the Fig. 2. DAB treatment

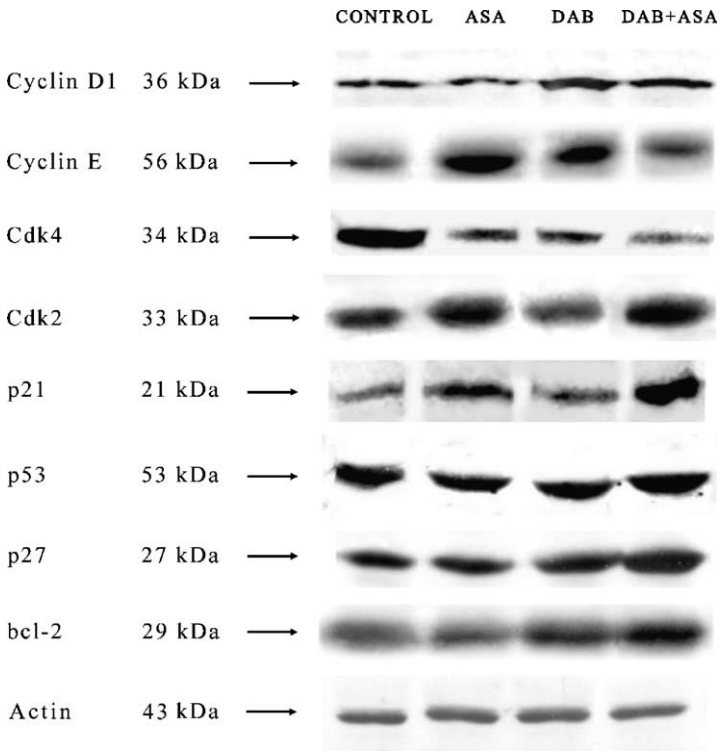

Fig. 2. Western blot analysis of cell cycle related proteins: cyclin D1, cyclin E, Cdk4, Cdk2, p21, p53, p27 and the anti-apoptotic protein bcl-2. Proteins were extracted from liver of control, ASA, DAB and DAB + ASA treated animals (three independent replicates). Equal quantities of proteins were loaded per lane. The proteins cyclin D1, cyclin E, Cdk4, Cdk2, p21, p53, p27 and bcl-2 were detected using polyclonal antibodies and visualised using ECL reagents. Intensity of bands was analysed with ImageMaster.

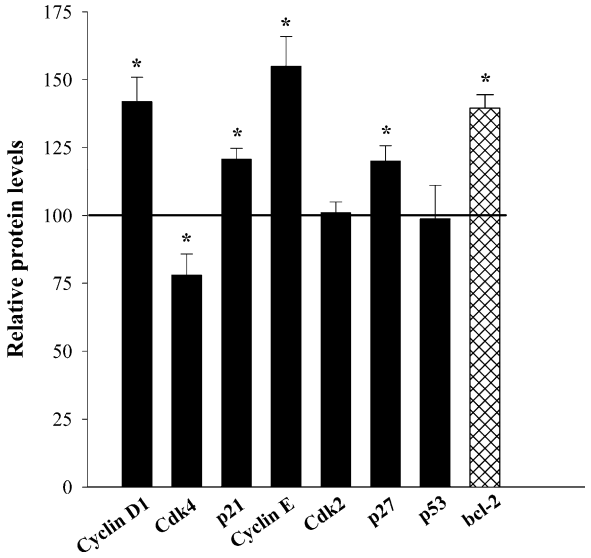

Fig. 3. Relative protein levels of cyclin D1, cyclin E, Cdk4, Cdk2, p21, p53, p27 and bcl-2 in DAB fed animals. Protein expression levels were expressed as a percentage of control values, obtained from Western blot analysis (Fig. 2). The black line represents the basal level. $^{*}P < 0.05$ vs. control group. Overexpression of cyclin D1 and cyclin E, deregulation of their Cdk partners and upregulation of the antiapoptotic bcl-2 protein induced by DAB treatment, is a common feature in proliferative states.

Fig. 4. DNA fragmentation analysis. DNA was extracted from liver of control and DAB treated animals (three independent replicates). The DNA damage provoked by DAB was visualised by DNA fragmentation in DAB treated animals.

increased the expression of cyclin D1 (42%) and cyclin E (55%) diminished Cdk4 (20%), but no alteration was provoked on Cdk2 expression. When the inhibitors of CDKs were investigated, over expression of p21 and p27 (21 and 20% over basal levels, respectively) was seen for both proteins while p53 remained unchanged. To determine the involvement of the cell death machinery in the carcinogenic process induced by DAB treatment, we evaluated the expression of the antiapoptotic protein bcl-2. DAB treatment highly induced bcl-2 expression.

In conclusion, we can assume that DAB treatment produced a deregulation in cell cycle progression which characterises the tumor process, reflected by over expression of cyclin D1 and cyclin E, down-regulation of Cdk4 and up-regulation of p21 and p27 expression (Fig. 3). The carcinogen administration also produced the induction of the antiapoptotic protein bcl-2. The damage caused by DAB was confirmed by the promotion of the hepatic endogenous DNA fragmentation, wich correlates with necrosis, in animals treated with this chemical carcinogen (Fig. 4).

3.2. *Effect of ASA on cell cycle related proteins*

The treatment with ASA produced an important attenuation in the induction of cyclin E provoked by DAB (reaching an expression level 17% over control animals) and an almost complete reversion of cyclin D1 enhancement to basal levels. Interestingly, p21 and p27 levels were increased when animals received both drugs. Meanwhile, the administration of ASA to DAB treated animals induced Cdk2 (29%) over basal levels (Figs. 2 and 5). These findings clearly showed that ASA treatment would drive to G1 arrest in response to DNA damage and oxidative stress.

ASA alone induced cyclin E, Cdk2 and p21 levels and diminished cyclin D1. The oncosupressor gene p53 remained unchanged and Cdk4 decreased respect to basal level. The antiapoptotic protein bcl-2 was not significantly modified (Fig. 2). Down-regulation of cyclin D1 associated with marked decreased Cdk4,

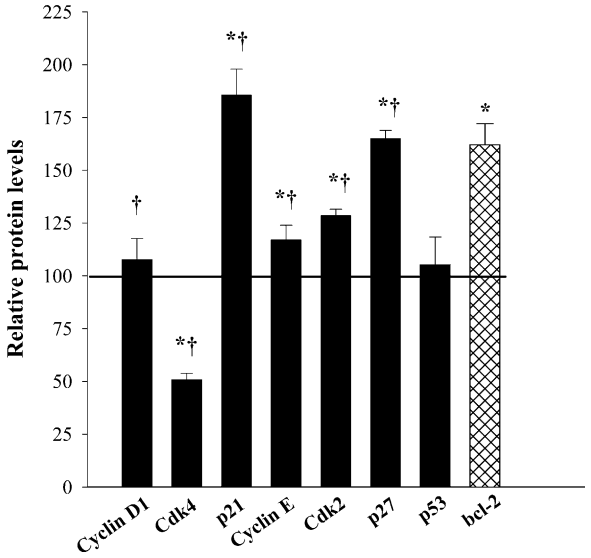

Fig. 5. Relative protein levels of cyclin D1, cyclin E, Cdk4, Cdk2, p21, p53, p27 and bcl-2 in DAB + ASA treated animals. Protein expression levels were expressed as a percentage of control values, obtained from Western blot analysis (Fig. 2). The black line represents the basal level. $^{*}P < 0.05$ vs. control group, $^{\dagger}P < 0.05$ vs. DAB group. An important increase in p21 (independent of p53) and p27 inhibitory proteins, a diminution in cyclin D1 and cyclin E levels, would drive to G1 arrest to repair the DNA damage provoked by the oxidative stress induced by the carcinogen administration. No changes in the antiapoptotic protein bcl-2 were detected.

reduced cell proliferation, modulation of cell cycle inhibitors, were previously reported for aspirin and other anti inflammatory drugs in cancer (Dihlmann, Siermann, & von Knebel Doeberitz, 2001).

3.3. HO-1 expression in chemically induced hepatocarcinogenesis and effect of the anti inflammatory agent ASA

We decided to investigate if the imbalance in the expression of the cell cycle regulators was related to the oxidative stress (Caballero, Gerez, Batlle, & Vazquez, 2002), provoked by the carcinogen. We analysed HO-1 expression which was increased a further 65% in DAB fed animals (Fig. 6), probably as consequence of the associated inflammation, a characteristic feature to the tumoral process, and previously detected in liver tissue samples of mouse intoxicated with DAB submitted to histological studies (Caballero et al., 2001). When DAB treated animals received the anti inflammatory agent ASA, HO-1 expression diminished reaching lower induction levels 23% over control (Fig. 6). No effect was observed on this protein expression in animals fed only with ASA (Fig. 6).

Our results suggest that the enhanced expression of HO-1 reflecting a response to ameliorate the increased oxidative stress induced by the carcinogen (Vazquez et al., 2002), could be attenuated by the administration of ASA through its well-known anti inflammatory properties also acting as an antioxidant agent (Hsu & Li, 2002).

4. Discussion

We have shown that DAB metabolisation through the microsomal hepatic fraction generates free radicals (Caballero et al., 2002). Oxidative injury may produce selective cell death and a compensatory increased in cell proliferation. This stimulus leads to unrepaired DNA damage resulting in the formation of a new mutation and, potentially, new initiated cells and/or enhancement of the selective clonal expansion of latent initiated cells (Klaunig et al., 1998). We demonstrated here that DAB treatment leads to deregulation of the cell cycle characteristic of the tumoral process. Expression of the cell cycle modulators at the G1-S boundary, p21, p27, p53, cyclin D1, cyclin E and their partner kinases: Cdk2 and Cdk4 was investigated in the chemically induced cancer liver model. Remarkable over expression of cyclin D1 and cyclin E with diminished Cdk4 levels and no alteration in Cdk2 showed an imbalance in the progression of the normal cell cycle of the organ. The expression of cell cycle inhibitors p21 and p27 was slightly increased, however, no variation was detected in p53 levels. Disruption of the regulatory system controlling G1 phase progression is a common event in human hepatocarcinogenesis (Hui, Makuuchi, & Li, 1998), and it was clearly evident in the in vivo model developed in our lab. Cyclin D1 and cyclin E are frequently overexpressed in cancer and it was recently reported that E type cyclins have an intrinsic Cdk independent function that is wholly unrelated to their capacity to promote activation of an established protein kinase (Gladden & Diehl, 2003).

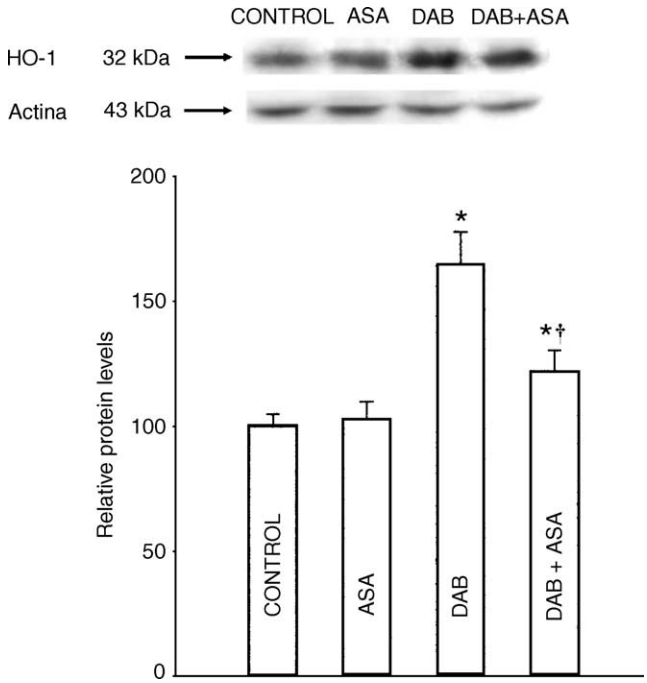

Fig. 6. Western blot analysis of HO-1. Proteins were extracted from liver of controls, ASA, DAB and DAB + ASA treated animals (three independent replicates). Equal quantities of proteins were loaded per lane. HO-1 protein was detected using a polyclonal antibody and visualised using ECL reagents. Intensity of bands was analysed with ImageMaster. HO-1 expression levels were expressed as a percentage of control values. $^{*}P < 0.05$ vs. control group, $^{\dagger}P < 0.05$ vs. DAB group. DAB provoked an overexpression of the protein which was partially reversed by ASA treatment.

Epidemiological studies have suggested that the use of aspirin is associated with a decreased incidence of human malignancies (Hsu & Li, 2002). It has been demonstrated that aspirin modulates the pro-inflammatory mediators and significantly reduces the size growth rate of transplanted tumors in mice (Kamate et al., 2002). Several studies suggest that aspirin and salicylates, inhibit cell growth, DNA and protein synthesis, and G1 to S progression in human colon adenocarcinoma cell lines (Ricchi et al., 1997). We found that the administration of ASA in DAB treated animals diminished cyclin D1 and cyclin E to near basal levels, in an attempt to restore their normal function. On the other hand, there was an important up-regulation of p21 and p27.

Agami and Bernards (2000) proposed that induction of G1 arrest in response to DNA damage is a two step process: a fast p53-independent initiation of G1 arrest mediated by cyclin D1 proteolysis and a slower maintenance of arrest resulting from increased p53 stability. Degradation of cyclin D1 is required to inhibit Cdk2 activity by distribution of p21 from Cdk4 complexes to inhibit Cdk2. Cyclin D1 degradation initiates a specific release of p21 from Cdk4 complexes, a process that culminates in a rapid increase of p21 associated with Cdk2 and inhibition of its kinase activity. This degradation is an essential component of the cellular response to genotoxic stress, in the absence of which the cells ability to deal with DNA damage is compromised (Agami & Bernards, 2000). Based on these findings, we propose that DNA damage induced by DAB intoxication could be repaired by ASA administration, due to longer arrest of cell cycle, as a consequence of diminution of cyclin D1 levels and up-regulation of p21 and p27, independent on p53. Inhibition of Cdk2 alone may be sufficient to achieve cell cycle arrest (Brooks et al., 1997). Marra, Tommaso Simoncini, James, and Liao (2000) reported that high doses of salicylates inhibited vascular smooth muscle cells proliferation and selectively unregulated $p21^{Waf1}$

and $p27^{Kip1}$ expression with greater inhibitory effect on Cdk2 compared with that on Cdk4/6, suggesting that salicylates blocked cell cycle progression from G1 to S phase. Although we cannot explain Cdk2 increased expression, however, we have observed the same Cdk2 expression profile in a model of regenerative hyperplasia (data not shown) and further investigations are required to explain these findings.

Salicylates and aspirin have been shown to inhibit the proinflammatory transcription factor NF-κB (Kopp & Ghosh, 1994), which play an important role in the regulation of genes involved in inflammation, cell differentiation, and cell growth (Siebenlist, Franzoso, & Brown, 1994). Aspirin did not affect p53 and had no effect on the apoptosis-controlling factors in human colorectal cancer cells (Menzel et al., 2002). ASA administration to DAB fed animals produced no significant changes in the expression of the antiapoptotic protein bcl-2 and the tumor suppressor p53, suggesting that apoptosis seems not to be involved in the protective mechanism of ASA.

Under our intoxication protocol with the carcinogen DAB, we have not observed morphological signals of apoptosis, under microscopic observation of histological sections of the liver (data not shown). Moreover, over expression of the antiapoptotic protein bcl-2 was observed in DAB animals, confirming the deficiency in the apoptotic machinery in this model.

Aberrant cell proliferation and abnormal expression of cell related proteins are observed in inflammatory disorders. Increased expression of HO-1, a stress responsive gene, corroborates the oxidative stress of the system that generates redox signals that would result in deregulation of growth control and imperfect DNA repair leading to carcinogenesis (Crosby et al., 2000). Up-regulation of HO-1 expression in DAB fed animals, as an endogenous antioxidant defense mechanism, would serve as a protective factor controlling the changes associated to the carcinogenic process.

Levels of HO-1 protein were diminished in the group DAB + ASA with respect to animals that received only the carcinogen, indicating that the anti inflammatory agent could be protecting the tissue from the oxidative injury and the inflammation provoked by DAB.

We reported herein p-21 up-regulation and a concomitant HO-1 diminution in the group DAB + ASA respect to DAB fed animals. In contrast, Inguaggiato et al. (2001) demonstrated that cellular over expression of HO-1 unregulated p21 in LLC-PK1 cells genetically engineered exhibit stable HO-1 over expression. This discrepancy might be due to the difference between the responses obtained by the use of cells culture and an in vivo experimental model where the whole organ and different tissue factors are involved.

Redox regulation of NF-κB is critical in the transcriptional control of the expression of TNFα and sustained TNFα induction is central to the pathogenesis of chronic liver disease (Tai et al., 2000). Recently, Tsukamoto (2002) has demonstrated that increased iron storage in hepatic macrophages is associated with accentuated and sustained NF-κB activation in a heme oxygenase-dependent manner. These data could justify the interrelationship between HO-1 role in inflammation and the rate of cell proliferation/apoptosis during the carcinogenesis and the protective effect of ASA in the tumoral process.

The complex interplay of heme oxygenase and the importance of defining the context in which the enzyme is induced must be considered. Therefore, to classify HO-1 as simply as a protective protein might to be an oversimplification (Zheng & Lavrovsky, 2000). Nevertheless, identification of HO-1 behaviour in an in vivo model of carcinogenesis is of significance and might have therapeutic or pharmacological implications. Once again, the usefulness of aspirin to ameliorate oxidative stress related diseases is worth to be considered.

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