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Persistent and Remodeling Hepatic Preneoplastic Lesions Present Differences in Cell Proliferation and Apoptosis, as well as in p53, Bcl-2 and NF-κB Pathways

Rogério Pietro Mazzantini, Aline de Conti, and Fernando Salvador Moreno*

Laboratory of Diet, Nutrition and Cancer, Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil

Abstract During rat hepatocarcinogenesis preneoplastic lesions (PNL) emerge which may persist (pPNL) and be sites of progress to cancer or suffer remodeling (rPNL) tending to disappear. Cellular and molecular mechanisms involved in both phenotypes are not sufficiently elucidated. pPNL and rPNL cellular proliferation and apoptosis were evaluated in rats submitted to the resistant hepatocyte (RH) model, and an adjusted growth index (AGI) was established. p53, Bcl-2, and NF-κB p65 subunit expression was evaluated by immunohistochemistry in pPNL and rPNL. p65 expression and NF-κB activation was evaluated by Western blot assays in whole livers. A lower number of BrdU-stained hepatocyte nuclei/mm^2 and higher number of apoptotic bodies (AB) per mm^2 were observed in remodeling compared to pPNL. Cytoplasmic p53 accumulation is related to increased hepatocarcinoma malignancy. We observed that 71.3% pPNL and 25.4% rPNL ($P < 0.05$) presented p53 staining in the cytoplasm. Similarly, 67.7% pPNL and 23.1 % rPNL ($P < 0.05$) presented increased Bcl-2 staining. Thirty-two percent pPNL and 15.6% rPNL ($P < 0.05$) presented p65 staining. Compared to normal rats, increase ($P < 0.05$) of hepatic p65 expression and NF-κB activation in rats submitted to the RH model was observed. In agreement to previous studies hepatic pPNL and rPNL differ regarding cell proliferation and apoptosis. Moreover, persistence and remodeling involve differences in p53, Bcl-2, and NF-κB pathways. These data point to molecular pathways that may direct preneoplastic lesions to spontaneously regress or to progress to cancer. J. Cell. Biochem. 103: 538–546, 2008.

Key words: hepatocarcinogenesis; preneoplastic lesions; persistence; remodeling; p53; bcl-2; NF-κB

Rat hepatocarcinogenesis models are valuable for the study of carcinogenesis [Su and Bannasch, 2003]. The development of hepatocarcinoma in these animals is suggested to be similar to human hepatocarcinogenesis [Bannasch et al., 2003]. The "resistant hepatocyte" (RH) model is well characterized and is able to induce preneoplastic lesions (PNL) which can be detected by γ-glutamyltranspeptidase (γGT) or glutathione *S*-transferase placental form (GST-P) markers. A typical and critical property of these hepatocyte foci and nodules is their capability of expressing one of two options: spontaneous remodeling to a normal appearing liver by the majority (95–98%) or persistence with cell proliferation and evolution to cancer by a small minority (2–5%).

These two kinds of hepatocyte foci and nodules show characteristic and consistent patterns of cell structure, biochemistry, histochemistry, and tissue architecture phenotypically quite different from liver at any stage in this normal development. During remodeling, the hepatocytes become less positive, in a patchy manner, for staining for some enzymes, such as γGT and GST-p presenting irregular borders and non-uniform staining [Enomoto and Farber, 1982; Tatematsu et al., 1983; Farber and Rubin, 1991; Imai et al., 1997]. PNL with regular borders and uniform staining for these markers are considered to be

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*Correspondence to: Fernando Salvador Moreno, Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Bloco 14, Av. Prof. Lineu Prestes 580, 05508-900, São Paulo, SP, Brazil. E-mail: rmoreno@usp.br

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persistent (pPNL) [Farber et al., 1988; Wood et al., 2002].

Redifferentiation was pointed as the mechanism involved in PNL remodeling [Enomoto and Farber, 1982; Tatematsu et al., 1983; Wood et al., 2002]. In addition, a lower labeling index and higher apoptotic index were observed in remodeling compared to pPNL [Enomoto and Farber, 1982; Schulte-Hermann et al., 1990].

Information on molecular mechanisms involved with PNL persistence or remodeling is scarce. p53 accumulation in the cytoplasm was related to the incapacity of hepatic PNL of rats submitted to the RH model to respond to genomic stress [Van Gijssel et al., 2000], and to an increase in malignancy of hepatocarcinomas [Heinze et al., 1999] and other neoplasias [Nikolaev et al., 2003]. Increase in Bcl-2 expression in basophilic PNL, considered to be aggressive [Su and Bannasch, 2003] was observed in mouse hepatocarcinogenesis [Christensen et al., 1999]. Hepatic NF-κB aberrant activation is an early event in the RH model suggesting loss of its regulation [Carrasco-Legleu et al., 2004; Espíndola et al., 2005; Simile et al., 2005].

In the present study, cell proliferation and apoptosis, nuclear and cytoplasmic p53 compartmentalization and Bcl-2 and NF-κB p65 subunit expression were evaluated specifically in hepatic pPNL and rPNL of rats submitted to the RH model.

MATERIALS AND METHODS

Chemicals

2-Acetylaminofluorene (2-AAF), 5-bromo-2-deoxyuridine (BrdU), 3,3-diaminobenzidine (DAB), diethylnitrosamine (DEN), and dimethylsulfoxide were purchased from Sigma (St. Louis). The commercial diet was purchased from Purina (Campinas, Brazil). Corn oil (CO) was Mazola® (São Paulo, Brazil). Polyclonal anti-placental glutathione *S*-transferase (GST-P) rabbit antibody was purchased from Medical and Biological Laboratories Co. (Tokyo, Japan). Immunohistochemical double-stain system kit, polyclonal anti-BrdU rat antibody, secondary biotinylated antibody and the streptavidin–biotin–peroxidase complex (StrepABComplex/HRP Duet, Mouse/Rabbit) were purchased from Dako (Glostrup, Denmark). BCA protein assay kits, N-PER™ and T-PER™ were purchased from Pierce (Rockford). Polyclonal anti-Bcl-2, anti-p53, and anti-p65 antibodies and secondary antibody conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz). ECL chemoluminescence kit and nitrocellulose membrane (Hybond™-C extra) were purchased from Amersham Biosciences (Piscataway). Other chemicals were of the highest available quality.

Animals and Experimental Protocol

Male F344 rats from the colony of the Faculty of Pharmaceutical Sciences, initially weighing 55–60 g, maintained in cages of four animals, at a constant temperature (22°C), with 12-h light-dark cycle and receiving water and commercial diet ad libitum, were used.

At the end of a 7-day acclimatization period, 20 F344 rats were randomly divided into 2 groups: 11 not submitted to any experimental procedure (normal [N] group), and 9 submitted to the RH model [Solt and Farber, 1976] modified by [Semple-Roberts et al., 1987] (RH group). Rats of RH received an intraperitoneal dose of DEN (20 mg/100 g body weight) for initiation. Two weeks later the animals received four intragastric doses of 2-AAF (2 mg/100 g of body weight), the first three on 3 consecutive days before partial (2/3) hepatectomy (PH) and the remaining (0.5 mg/100 g of body weight) 4 days after PH. Two hours before sacrifice the rats received a single i.p. injection of BrdU (10 mg/100 g of body weight) dissolved in dimethylsulfoxide and saline (1:3 v/v). Animals were sacrificed 6 weeks after initiation with DEN. The study was conducted in accordance with NIH guidelines for the care and use of laboratory animals.

Immunohistochemistry for GST-p, BrdU, p53, Bcl-2, and p65

Representative fragments of each liver lobe were fixed in Carnoy's solution prepared as previously described [Espíndola et al., 2005]. Serial histologic sections of the liver samples were also processed to be submitted to immunohistochemical reactions in order to detect PNL (foci/nodules) positive for GST-P and hepatocytes positive for BrdU, according to the instruction of the Dako immunohistochemical double-stain kit. The first used primary antibody was the monoclonal anti-BrdU antibody (1:400), its chromogenic substrate being diaminobenzidine. The second used primary antibody was the polyclonal anti-GST-P antibody (1:500)

to stain pPNL or rPNL with Fast Red as chromogenic substrate. Hepatocyte foci and nodules staining uniformly and non-uniformly for GST-P were classified as pPNL and rPNL, respectively [Wood et al., 1999] (Fig. 1A,B). They were measured with the Image Pro Plus program (Media Cybernetic) using an Olympus photomicroscope (Japan) connected to a microcomputer. Data were expressed as GST-P positive number (n/cm^2) and area (mm^2), and % liver section occupied by these PNL. Reading of the BrdU-stained hepatocyte nuclei was performed by scanning the whole slide and the result was expressed as the number of BrdU-stained cells/mm^2 of normal tissue, pPNL, rPNL or surrounding area.

Other serial histologic sections were prepared for immunohistochemical staining with anti-p53, anti-Bcl-2, and anti-p65 antibodies, using the ImmunoCruz Staining Systems kit, according to the manufacturer's instruction. The diluted primary antibodies (1:100) were incubated for 2 h. Coloring was developed with DAB substrate (Sigma). The slides were counterstained with hematoxylin. Specificity of staining was controlled by subtracting the primary antibody or by substituting it for non-immunized goat serum. In all cases this resulted in absence of staining. The reprodutibility of the methods was checked by staining more than one slide from the same animal, obtaining consistent results. In addition a histologic section of epidermoid cell carcinoma (DakoCytomation, Carpinteria) was used as positive control for p53.

Quantitative Evaluation of Immunohistochemical p53, Bcl-2, and p65 Staining

The cytoplasmic staining of p53 in apparently normal hepatocytes surrounding the preneoplastic cells was undetectable in all rats, while Bcl-2 or p65 surrounding staining was low. We compared staining of p53, Bcl-2, and p65 between the different rats submitted to the RH protocol and found that staining was equal in all rats. Therefore, we could use the cytoplasmic or whole cell staining as an internal reference. The intensity of staining in the cytoplasm (p53 and Bcl-2) or whole cell (p65) of preneoplastic cells was classified as higher than (positive p53, Bcl-2, or p65 staining PNL) or equal to (negative p53, Bcl-2, or p65 staining PNL) the reference staining in the cytoplasm or whole cell of surrounding hepatocytes. A PNL was classified as positive for p53, Bcl-2, or p65 staining if 50% or more of the cells concerned were stained more intensively than the staining in surrounding hepatocytes [Van Gijssel et al., 2000]. To verify if the PNL were pPNL or rPNL, all the immunohistochemical evaluation analyses of p53, Bcl-2, and p65 staining were made

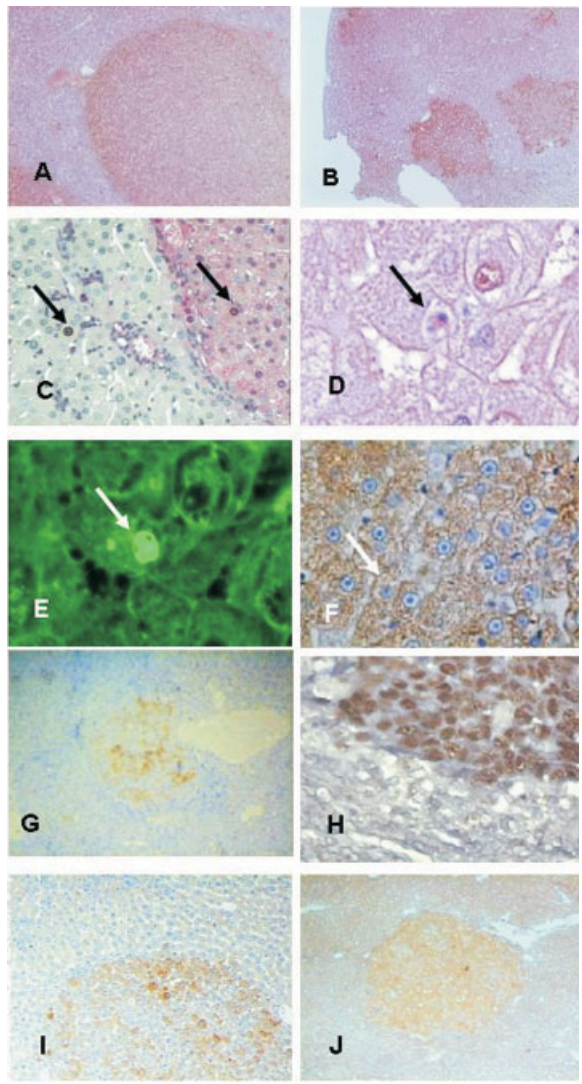

Fig. 1. Persistent (**A**) and remodeling (**B**) GST-P positive PNL. Hepatic histologic section of F344 rat submitted to the RH model showing immunohistochemical double staining (**C**) for positive pPNL GST-P (red area) and for BrdU (hepatocytes nucleous indicated by arrows outside and inside pPNL); 40X objective. Example of apoptotic body detected in hepatic histologic section, stained with hematoxilin and eosin (H&E), of F344 rat submitted to the RH model by transmitted light (**D**) and by fluorescence microscopy (**E**). Arrow indicates pPNL hepatocyte cytoplasmatic immunostaining for p53 (**F**). p53 immunostaining (**G**). p53 nuclear immunostaining in squamous carcinoma cells (positive control) (**H**). Bcl-2 immunostaining (10X objective) (**I**). p65 immunostaining (10X objective) (**J**).

comparing the serial histological sections with those marked for GST-P.

Apoptosis Evaluation

For the evaluation of apoptosis a fluorescence microscopy method [Stinchcombe et al., 1995; Ong et al., 2005] was used for histologic sections of N and RH group rats, the latter being immunostained for GST-P, as previously described, and counterstained with eosin. The epifluorescence microscope (Nikon, Japan) was used for quantification of the number of AB both in the areas of normal tissues surrounding PNL as well as in them, allowing to discriminate between pPNL and rPNL. The total area of histologic sections was analyzed with a 40X objective and the results were confirmed according to classical morphologic criteria described in the literature, changing the epifluorescence system to transmitted light [Grasl-Kraupp et al., 1994]. Results were expressed as number of AB/mm^2 of total area of N group rat liver section or areas of pPNL, rPNL or those surrounding them in RH group animals.

Adjusted Growth Index (AGI) Calculation

To access the degree to which cellular proliferation and apoptosis changes may affect net growth potential of pPNL and rPNL, and (AGI) was established. AGI was calculated as the number of BrdU-positive hepatocytes/mm^2 divided by the number of AB/mm^2 of pPNL, rPNL, and normal or surrounding area. According to this formula, when the number of BrdU-positive hepatocytes/mm^2 (numerator value) is greater than the number of AB/mm^2 (denominator value), the AGI will be greater than one (predominance of cell proliferation over apoptosis). On the other hand, and AGI smaller than one indicate the opposite condition (predominance of apoptosis over cell proliferation). During the RH model, S phase of cell cycle present a length of 5.9 h [Rotstein et al., 1986] whereas AB are only visible for ~3 h in the liver, before they are completely degraded by other cells [Wood et al., 1999].

p65 Western Blot Analysis

The method described by Espíndola et al. (2005) was used. Basically, total and nuclear protein extracts were prepared from liver samples of the experimental rats, previously stored at −78°C, using the T-PER^{TM} or N-PER^{TM} reagent, in order to evaluate NF-κB p65 subunit expression and NF-κB activation, respectively. Control of the relative amount of the protein of interest was made by previous staining of the nitrocellulose membrane with Coomassie blue [Tao et al., 2002; Espíndola et al., 2005; Ong et al., 2005].

Statistical Analysis

For the statistical analysis the SigmaStat program (Statwin Software—Copyright 1992–1994) was used. When indicated the used tests were Fisher's exact and Student's *t*-tests. Level of significance was considered $P < 0.05$.

RESULTS

Morphometric Analysis

Table I shows the results of morphometric quantification of number, mean area, and percent of the histologic section occupied by GST-P positive PNL in the liver of rats submitted to the RH model. No statistically significant difference ($P > 0.05$) between the number of pPNL and rPNL was observed. pPNL presented a greater ($P < 0.05$) mean area and occupied a higher ($P < 0.05$) percent of section area than rPNL.

Cell Proliferation and Apoptosis

Immunohistochemical double staining was used for BrdU-positive cell count, inside and outside pPNL (Fig. 1C) and rPNL. Table II shows the results of mean BrdU-positive hepatocyte number of the histologic sections of N and

TABLE I. Morphometric Analysis of GST-P Positive PNL of F344 Rats Submitted to the RH Model of Hepatocarcinogenesis*

PNL type	Mean number of PNL/cm^2	Mean area of PNL (mm^2)	% Area of section occupied by PNL
Total (pPNL + rPNL)	55.74 ± 16.65	0.60 ± 0.38	34.45 ± 25.01
pPNL	31.51 ± 11.72	0.84 ± 0.50[a]	29.18 ± 24.60[a]
rPNL	24.22 ± 8.01	0.24 ± 0.11	5.45 ± 1.83

*Values are means ± SD.
[a]Statistically significant difference compared to rPNL (Student's *t*-test, $P < 0.05$); n = 9.

TABLE II. Mean Number of BrdU-Stained Hepatocytes/mm^2 and of Hepatic Apoptotic Bodies/mm^2 and AGI of Rats Submitted and Not Submitted to the RH Model*

		RH model			
	Normal	Surrounding	Total PNL (pPNL + rPNL)	pPNL	rPNL
BrdU	1.44 ± 0.92	1.52 ± 1.13	1.99 ± 0.87[a]	2.26 ± 1.11[a]	1.32 ± 0.79
Apoptosis	0.54 ± 0.40	0.74 ± 0.36	1.83 ± 0.69[b]	1.33 ± 1.07[a]	2.39 ± 1.34[b,c]
AGI	3.22 ± 2.82[a]	2.44 ± 1.72[a]	1.97 ± 1.21[a]	2.83 ± 2.03[a]	0.80 ± 0.72

*Values are means ± SD.
[a]Statistically significant difference compared to rPNL (Student's t-test, $P < 0.05$); n = 9 submitted to the RH model and 11 normal animals.
[b]Statistically significant difference compared to normal liver tissue (Student's t-test, $P < 0.05$).
[c]Statistically significant difference compared to surrounding (Student's t-test, $P < 0.05$).

RH groups. Total PNL and pPNL presented a higher mean number of BrdU-positive hepatocytes compared to their respective surroundings and to N group hepatic tissue, although these differences were not significant. These results agree with the information that during hepatocarcinogenesis cell proliferation increases [Espíndola et al., 2005; Ong et al., 2005] and that pPNL present elevated levels of labeling index compared to normal liver [Rotstein et al., 1986]. Total PNL and the pPNL presented a higher ($P < 0.05$) mean number of BrdU-positive hepatocytes compared to rPNL. rPNL mean number of BrdU-positive hepatocytes was similar to that of N group hepatic tissue.

Table II shows the results of mean number of AB (Fig. 1D,E) of the histologic sections of N and RH groups. Total PNL, pPNL, and rPNL of the RH group presented a higher mean number of AB compared to their surroundings and to N group, although the differences reached statistical significance ($P < 0.05$) only when comparing total PNL with normal rat tissue and rPNL with their surrounding and normal rat tissue. These results agree with the information that during hepatocarcinogenesis apoptosis increases [Talos et al., 2005; Zaika et al., 1999] and that pPNL present elevated levels of cell death compared to normal liver [Rotstein et al., 1986]. pPNL presented lower ($P < 0.05$) mean number of AB compared to rPNL.

As previously observed [Enomoto and Farber, 1982; Schulte-Hermann et al., 1990], a lower number of BrdU-stained hepatocyte nuclei/mm^2 and higher number of AB/mm^2 were observed in remodeling compared to pPNL.

AGI

No differences ($P > 0.05$) were observed between normal, surrounding, total and pPNL tissues regarding AGI. Compared to rPNL, all these tissues presented greater ($P < 0.05$) AGI.

Immunostaining for p53

Cytoplasmic p53 staining was observed only in PNL hepatocytes, either in pPNL (Fig. 1F,G) or in rPNL. In the positive control was observed a nuclear staining for p53 (Fig. 1H). Figure 2A shows that a higher ($P < 0.05$) percentage of pPNL presented cytoplasmic p53 staining than rPNL.

Immunostaining for Bcl-2

PNL presented a more intense cytoplasmic Bcl-2 staining than the surrounding area

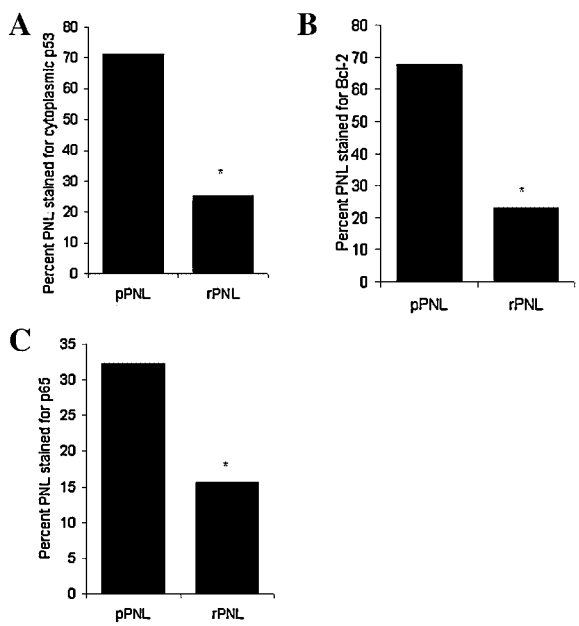

Fig. 2. Percent pPNL and rPNL immunohistochemically stained for cytoplasmatic p53 (**A**), Bcl-2 (**B**), p65 (**C**). n = 9 rats submitted to the RH model. Abbreviations are: pPNL, persistent PNL; rPNL, remodeling PNL. *$P < 0.05$ as compared to pPNL (Fisher's exact test).

(Fig. 1I). While pPNL Bcl-2 staining was intense, rPNL staining was weak and similar to the surrounding area. Figure 2B shows that a higher ($P < 0.05$) percentage of pPNL presented cytoplasmic Bcl-2 staining than rPNL.

Immunostaining for p65

PNL presented a more intense p65 staining than the surrounding area (Fig. 1J). Figure 2C shows that a higher ($P < 0.05$) percentage of pPNL presented p65 staining than rPNL.

NF-κB Activation and Its p65 Subunit Expression

p65 activation and expression [Carrasco-Legleu et al., 2004; Espíndola et al., 2005] were evaluated by the presence of p65 in nuclear and total protein extracts from whole livers, respectively. Figure 3 shows p65 immunoblots performed with whole liver (nodules + non-nodular surrounding tissues) nuclear or total protein extracts from N and RH groups (A), as well as their densitometric analysis (B). Compared to N group, RH group presented approximately a 20-fold increase ($P < 0.05$) of nuclear p65 levels and about a 2.5-fold increase ($P < 0.05$) of p65 expression.

DISCUSSION

In the RH model total (persistent + remodel-remodeling) GSTP + preneoplastic lesions cell proliferation peak occur at day 4 after partial hepatectomy and decrease to levels similar to the surrounding from day 14 to 21 [Wood et al., 1999]. Considering that in our study, cell proliferation was measured at day 23 after partial hepatectomy it would be expected that no differences would be observed between pPNL AGI and normal and surrounding AGI. In this study, hepatic GST-P positive pPNL presented an AGI over 1, indicating predominance of cell proliferation as related to apoptosis [Kong and

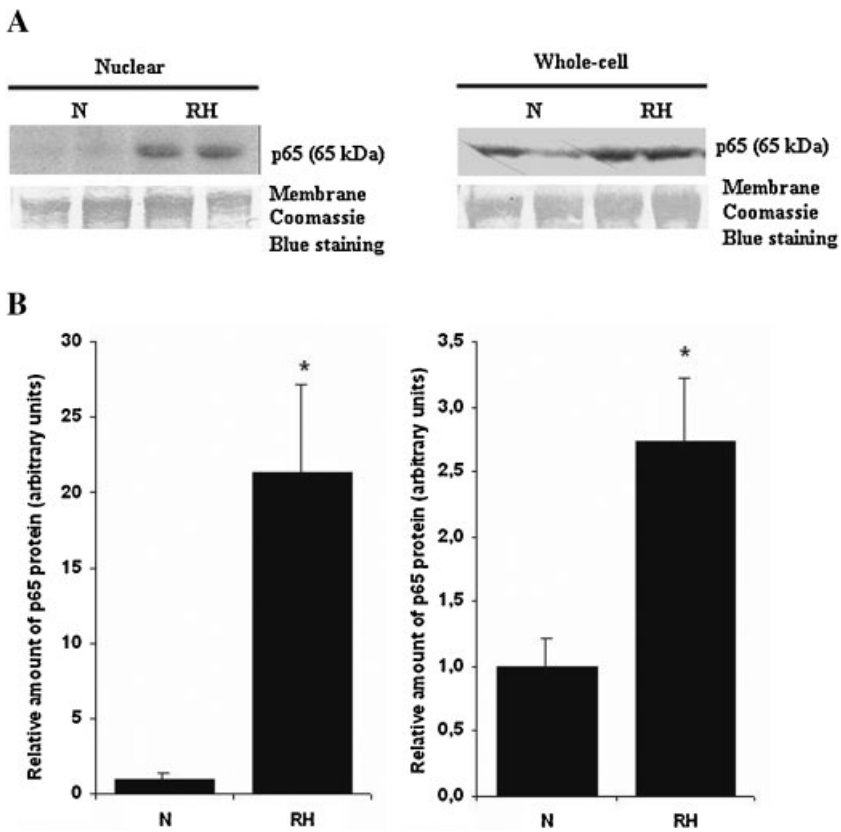

Fig. 3. NF-κB p65 subunit Western blot analysis performed with nuclear and whole-cell proteins extracted from whole livers of normal F344 rats (N) and animals submitted to the RH model (RH). Representative samples from two animals of each group. Below, membrane Coomassie blue staining for protein equal loading control (**A**). Quantification of p65 in nuclear and whole-cell proteins extracted from whole livers of normal F344 rats (N) and animals submitted to the RH model (RH). A total of four (N group) and six (RH group) animals were analyzed in two independent experiments. Results are expressed in relation to normal animals' p65 levels considered to be 1 (**B**). Values are means ± SD. *Statistics by Student's *t*-test: significant differences ($P < 0.05$) when compared to N group.

Ringer, 1996]. rPNL, instead, presented an AGI lower than 1 indicating predominance of apoptosis as compared to cell proliferation. Although AGI can be useful, it must be carefully analyzed since it could lead to misinterpretation of the results. BrdU once in the nucleus has much longer half-life. Even after the cell divides the label is still there, albeit gets diluted with progressive cell division. But still the cell is counted as a BrdU-positive cell. The apoptotic cell on the other hand has a very short half-life of 3–5 h [Bursch et al., 1990]. Because of this, there could be a bias towards an AGI over 1. The fact that rPNL presented an AGI lower than 1 reinforces the observation that apoptosis predominates as related to cell proliferation. The results of this study are in agreement with previous observations in rats also submitted hepatocarcinogenesis models that pPNL presented a higher cell proliferation and a lower apoptotic index when compared to rPNL [Enomoto and Farber, 1982; Schulte-Hermann et al., 1990].

Nuclear p53 localization has been considered essential for its growth-suppressing activity in late G_1 [Shaulsky et al., 1991] although recently it has been described that mitochondrially targeted p53 has also tumor suppressor activities stimulating apoptosis in vivo without the need of its translocation to the nucleus [Talos et al., 2005]. Tumors might abrogate the transactivating function of p53 by inhibiting its access to the nucleus rather than by gene mutation [Moll et al., 1995]. Thus, pathologic accumulation of cytoplasmic wild-type p53 has been related to alteration in differentiation, increase in malignancy, tumor metastasis and poor prognosis of hepatocarcinomas [Heinze et al., 1999] and other cancers [Moll et al., 1995; Jimenez et al., 1999]. p53 regulation is effected by different control mechanisms of nuclear-cytoplasmic shuttling [Fabbro and Henderson, 2003], such as its interaction with proteins which would inhibit its translocation or promote its export from the nucleus [Kim et al., 2000; Nikolaev et al., 2003].

Normally, the level of p53 protein is tightly regulated and not detectable by immunohistochemistry. Phenotypes with constitutive p53 nuclear or cytoplasmic accumulation are considered to be valuable systems in elucidating mechanisms that regulate p53 turnover [Zaika et al., 1999]. Thus, a previous study in PNL-bearing rats challenged with DEN has documented that PNL has an attenuated p53 nuclear response. It was suggested that this may facilitate clonal expansion of PNL under stress induced by DNA-damaging chemicals [Lennartsson et al., 1988]. In rats submitted to the RH model X-ray-induced genotoxic stress resulted in p53 accumulation in the cytoplasm of GST 7-7-positive PNL hepatocytes and in the nucleus of surrounding hepatocytes [Van Gijssel et al., 2000]. However, in this study the authors did not distinguish pPNL from rPNL regarding cytoplasmic p53 accumulation in contrast to our study. We observed for the first time that a greater proportion of pPNL presented aberrant cytoplasmic p53 accumulation compared to rPNL. The pPNL phenotype could be related to alterations in p53 translocation to the nucleus. This could be related to an increased genomic instability early in hepatocarcinogenesis, which not only sustains clonal expansion, but also increases the probability of further genetic changes leading towards malignant transformation [Van Gijssel et al., 2000]. The rPNL phenotype could be related to a normalization of the p53 pathway. Indeed, accumulation of cytoplasmic p53 was more frequent in undifferentiated neuroblastomas than in those differentiated, suggesting that the p53 alteration is potentially reversible and may cause reconstitution of functional p53 [Moll et al., 1995]. It would be interesting to evaluate in the RH model if chemopreventive compounds would interfere with p53 localization in pPNL and rPNL.

Increased expression of Bcl-2 was previously observed in PNL and hepatic cancers during non-genotoxic hepatocarcinogenesis in mice [Christensen et al., 1999] and in PNL of Wistar rats submitted to the RH model [Van Gijssel et al., 2000]. However, in this last study the authors did not distinguish pPNL from rPNL regarding Bcl-2 expression in contrast to our study. We observed for the first time that a greater proportion of pPNL presented increased Bcl-2 expression compared to rPNL. While pPNL Bcl-2 staining was intense, rPNL staining was weak and similar to the surrounding area. This suggests that increased Bcl-2 expression is involved with persistence and that an increase in apoptosis in rPNL could be attributed to a return of Bcl-2 expression to levels similar to those of normal tissue. Bcl-2 gene contains a negative response element through which p53 may transcriptionally downregulate

its expression [Miyashita et al., 1994]. The fact that p53 was accumulated in the cytoplasm of pPNL could result in absence of its nuclear inhibitory effects, increasing Bcl-2 expression.

NF-κB activation is an early event during hepatocarcinogenesis [Carrasco-Legleu et al., 2004; Espíndola et al., 2005; Simile et al., 2005]. This study confirms this observation and shows an increased NF-κB p65 subunit expression in the liver of rats submitted to the RH model. It was observed for the first time that a greater proportion of pPNL presented increased NF-κB p65 expression compared to rPNL. In this study we observed increased levels of p53 in the cytoplasm, but not in the nucleus of pPNL. A persistent nuclear NF-κB could reduce p53 protein nuclear stabilization [Tergaonkar et al., 2002]. Concomitant alterations in both pathways could be related to pPNL phenotype. That a decreased proportion of rPNL presented increased NF-κB p65 subunit expression suggests that remodeling would also be followed by a normalization of its expression.

Based on our previous investigations with the RH model [Espíndola et al., 2005; Ong et al., 2005] we have chosen to study pPNL and rPNL 6 weeks after DEN initiation during a phase in which the proportion of these PNL is similar (around 50%). This allowed us to analyze an adequate number of each PNL in order to establish differences between them. It is important to stress that the remodeling process is a continuum and takes place over several weeks, and the majority of PNL considered persistent at this time point will remodel. Thus, in future studies it would be interesting to evaluate if the few pPNL that progress to cancer would be the ones presenting simultaneous alterations in p53, Bcl-2, and NF-κB pathways.

In agreement to previous studies hepatic pPNL and rPNL differ regarding cell proliferation and apoptosis. Moreover, persistence and remodeling involve differences in p53, Bcl-2, and NF-κB pathways. These new data point to molecular pathways that may direct preneoplastic lesions to spontaneously regress or to progress to cancer.

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REFERENCES

Bannasch P, Haertel T, Su Q. 2003. Significance of hepatic preneoplasia in risk identification and early detection of neoplasia. Toxicol Pathol 31:34–139.

Bursch W, Paffe S, Putz B, Barthel G, Schulte-Hermann R. 1990. Determination of the lenght of the histological stages of apoptosis in normal liver and in altered foci of rats. Carcinogenesis 11:847–853.

Carrasco-Legleu CE, Márquez-Rosado L, Fattel-Fazenda S, Arce-Popoca E, Pérez-Carreón JI, Villa-Treviño S. 2004. Chemoprotective effect of caffeic acid phenethyl ester on promotion in a medium-term rat hepatocarcinogenesis assay. Int J Cancer 108:488–492.

Christensen JG, Romach EH, Healy LN, Gonzales AJ, Anderson SP, Malarkey DE, Corton JC, Fox TR, Cattley RC, Goldsworth TL. 1999. Altered Bcl-2 family expression during non-genotoxic hepatocarcinogenesis in mice. Carcinogenesis 20:1583–1590.

Enomoto K, Farber E. 1982. Kinetics of phenotypic maturation of remodeling of hyperplastic nodules during liver carcinogenesis. Cancer Res 42:2330–2335.

Espíndola RM, Mazzantini RP, Ong TP, de Conti A, Heidor R, Moreno FS. 2005. Geranylgeraniol and beta-ionone inhibit hepatic preneoplastic lesions, cell proliferation, total plasma cholesterol and DNA damage during the initial phases of hepatocarcinogenesis, but only the former inhibits NF-kappaB activation. Carcinogenesis 26:1091–1099.

Fabbro M, Henderson BR. 2003. Regulation of tumor suppressors by nuclear-cytoplasmic shuttling. Exp Cell Res 282:59–69.

Farber E, Rubin H. 1991. Cellular adaptation in the origin and development of cancer. Cancer Res 51:2751–2761.

Farber E, Chen ZY, Harris L, Lee G, Rinaudo JS, Roomi WM, Rotstein J, Semple E. 1988. The biochemical-molecular pathology of the stepwise development of liver cancer: New insights and problems. In: Bannasch D, Keppler GW, editors. Liver cell carcinoma Falk Symposium 51. Dordrecht: Kluwer Academic Publishers. pp 273–291.

Grasl-Kraupp B, Bursch W, Ruttkay-Nedecky B, Wagner A, Lauer B, Shulte-Herrmann R. 1994. Food restriction eliminates preneoplastic cells through apoptosis and antagonizes carcinogenesis in rat liver. Proc Natl Acad Sci USA 91:9995–9999.

Heinze T, Jonas S, Karsten A, Neuhaus P. 1999. Determination of the oncogenes p53 and C-erb B2 in the tumour cytosols of advanced hepatocellular carcinoma (HCC) and correlation to survival time. Anticancer Res 19:2501–2503.

Imai T, Masui T, Ichinose M, Nakanishi H, Yanai T, Masegi T, Muramatsu M, Tatematsu M. 1997. Reduction of glutathione S-transferase P-form mRNA expression in remodeling nodules in rat liver revealed by in situ hybridization. Carcinogenesis 18:545–551.

Jimenez G, Khan S, Stommel J, Wahl G. 1999. p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. Oncogene 8: 7656–7665.

Kim IS, Kim DH, Han SM, Chin MU, Nam HJ, Cho HP, Choi SY, Song BJ, Kim ER, Bae YS, Moon YH. 2000. Truncated form of importin alpha identified in breast cancer cell inhibits nuclear import of p53. J Biol Chem 275:23139–23145.

Kong J, Ringer DP. 1996. Quantitative analysis of changes in cell proliferation and apoptosis during preneoplastic and neoplastic stages of hepatocarcinogenesis in rat. Cancer Lett 105:241–248.

Lennartsson P, Hogberg J, Stenius U. 1988. Wild-type p53 expression in liver tissue and in enzyme-altered foci: An in vivo investigation on diethylnitrosamine-treated rats. Carcinogenesis 19:1231–1237.

Miyashita T, Harigai M, Hanada M, Reed JC. 1994. Identification of a p53-dependent negative response element in the Bcl-2 gene. Cancer Res 54:3131–3135.

Moll UM, Laquagliat M, Benard J, Riou G. 1995. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc Natl Acad Sci USA 92:4407–4411.

Nikolaev AY, Li M, Puskas N, Qin J, Gu W. 2003. Parc: A cytoplasmic anchor for p53. Cell 112:29–40.

Ong TP, Heidor R, de Conti A, Dagli ML, Moreno FS. 2005. Farnesol and geraniol chemopreventive activities during the initial phases of hepatocarcinogenesis involve similar actions on cell proliferation and DNA damage, but distinct actions on apoptosis, plasma cholesterol and HMGCoA reductase. Carcinogenesis 27:1194–1203.

Rotstein J, Sarma DSR, Farber E. 1986. Sequential alterations in growth and cell dynamics of rat hepatocytes in early precancerous steps in hepatocarcinogenesis. Cancer Res 46:2377–2385.

Schulte-Hermann R, Timmermann-Trosiener L, Barthel G, Bursch W. 1990. DNA synthesis, apoptosis, and phenotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion. Cancer Res 50:5127–5135.

Semple-Roberts E, Hayes MA, Armstrong D, Becker RA, Racz WJ, Farber E. 1987. Alternative methods of selecting rat hepatocellular nodules resistant to 2-acetylaminofluorene. Int J Cancer 40:643–645.

Shaulsky G, Goldfinger N, Tosky M, Levine AJ, Rotter V. 1991. Nuclear localization is essential for the activity of p53 protein. Oncogene 6:2055–2065.

Simile MM, Pagnan G, Pastorino F, Brignole C, De Miglio MR, Muroni MR, Asara G, Frau M, Seddaiu MA, Calvisi DF, Feo F, Ponzoni M, Pascale RM. 2005. Chemopreventive N-(4-hydroxyphenyl)retinamide (fenretinide) targets deregulated NF-κB and Mat1A genes in the early stages of rat liver carcinogenesis. Carcinogenesis 26: 417–427.

Solt DT, Farber E. 1976. New principle for the analysis of chemical carcinogenesis. Nature 263:702–703.

Stinchcombe S, Buchmann A, Bock KW, Schwarz M. 1995. Inhibition of apoptosis during 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated tumour promotion in rat liver. Carcinogenesis 16:1271–1275.

Su Q, Bannasch P. 2003. Relevance of hepatic preneoplasia for human hepatocarcinogenesis. Toxicol Pathol 31:126–133.

Talos F, Petrenko O, Mena P, Moll UM. 2005. Mitochondrially targeted p53 has tumor suppressor activities in vivo. Cancer Res 65:9971–9981.

Tao L, Kramer PM, Wang W, Yang S, Lubet RA, Steele VE, Pereira MA. 2002. Altered expression of c-myc, p16 and p27 in rat colon tumors and its reversal by short-term treatment with chemopreventive agents. Carcinogenesis 23:1447–1454.

Tatematsu M, Nagamine Y, Farber E. 1983. Redifferentiation as a basis for remodeling of carcinogen-induced hepatocyte nodules to normal appearing liver. Cancer Res 43:5049–5058.

Tergaonkar V, Pando M, Vafa O, Whal G, Verma I. 2002. p53 stabilization is decreased upon NFκB activation: A role for NFκB in acquisition of resistance to chemotherapy. Cancer Cell 1:493–503.

Van Gijssel HE, Ohlson LCE, Torndal UB, Mulder GJ, Eriksson LC, Porsch-Hällström I, Meerman JHN. 2000. Loss of nuclear p53 protein in preneoplastic rat hepatocytes is accompanied by Mdm2 and Bcl-2 overexpression and by defective response to DNA damage in vivo. Hepatology 32:701–710.

Wood GA, Sarma DS, Archer MC. 1999. Resistance to the promotion of glutathione S-transferase 7-7-positive liver lesions in Copenhagen rats. Carcinogenesis 20: 1169–1175.

Wood GA, Korkola JE, Archer MC. 2002. Tissue-specific resistance to cancer development in the rat: Phenotypes of tumor-modifier genes. Carcinogenesis 23:1–9.

Zaika A, Marchenko N, Moll UM. 1999. Cytoplasmically "sequestered" wild type p53 protein is resistant to mdm2-mediated degradation. J Biol Chem 274:27474–27480.