All-trans and 9-cis retinoic acids, retinol and β-carotene chemopreventive activities during the initial phases of hepatocarcinogenesis involve distinct actions on glutathione S-transferase positive preneoplastic lesions remodeling and DNA damage

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Chemopreventive activities of all-trans retinoic acid (ATRA), 9-cis retinoic acid (9cRA), retinol (ROL) and β-carotene (βC) were evaluated during hepatocarcinogenesis. Rats received 1 mg/100 g body wt ATRA (ATRA group), 9cRA (9cRA group), ROL (ROL group), 7 mg/100 g body wt βC (βC group) or corn oil (CO group, controls). Hepatocyte nodule incidence was reduced (P < 0.05) in βC group (46%), but not (P > 0.05) in ATRA (92%), 9cRA (92%) and ROL (82%) groups, compared with the CO group (100%). Multiplicity of these preneoplastic lesions (PNL) was different (P < 0.05) between CO group (44 ± 9) and 9cRA (11 ± 4), ROL (7 ± 3) and βC (4 ± 2) groups, except for ATRA group (27 ± 9; P > 0.05). Number/mm² liver section, mean area (mm²) and percent liver section area occupied by total (persistent + remodeling) placent al glutathione S-transferase (GST-P) positive PNL was reduced (P < 0.05) in ATRA (107 ± 13; 0.12 ± 0.06; 13.9 ± 3.9), 9cRA (71 ± 12; 0.12 ± 0.06; 6.8 ± 2.2), ROL (96 ± 13; 0.11 ± 0.22; 6.8 ± 2.0) and βC (106 ± 13; 0.08 ± 0.03; 10.8 ± 2.5) groups compared with CO group (166 ± 14; 0.18 ± 0.09; 28.6 ± 5.2). Percent of remodeling GST-P positive PNL was increased (P < 0.05) in 9cRA (92 ± 1), ROL (96 ± 1) and βC (93 ± 1) groups, but not (P > 0.05) in ATRA group (90 ± 2), compared with the CO group (86 ± 1). Compared with the CO group, all groups present in PNL reduced (P < 0.05) cell proliferation and no differences (P > 0.05) in apoptosis. DNA damage [comet (µm)] was reduced (P < 0.05) in ROL (87.9 ± 2.6) and βC (89.2 ± 4.0) groups, but not in ATRA (94.8 ± 4.1) and 9cRA (94.2 ± 1.5) groups, compared with the CO group (100.4 ± 3.9). ATRA, 9cRA, ROL and βC presented chemopreventive activities against hepatocarcinogenesis. These involve inhibition of cell proliferation, but not induction of apoptosis. Increased remodeling of GST-P positive PNL relates to 9cRA, ROL and βC actions, while inhibition of DNA damage relates to ROL and βC actions.

Introduction

In view of the limited treatment and negative prognosis of liver cancer, preventive control approaches, notably chemoprevention, have been emphasized (1). Polypropenoic acid, a synthetic retinoid, prevents secondary tumors in patients with hepatocellular carcinoma (HCC) (2) and increases their survival (3), and hepatic retinoid content decreases early in carcinogenesis (4). This suggests that retinol (ROL; vitamin A) and its natural analogs may have also a place in human liver cancer control (5).

ROL and βC, a pro-vitamin A, presented protective actions in different phases of hepatocarcinogenesis models including that of the resistant hepatocyte (RH) (1.4–6). However, there are controversies regarding the use in hepatocarcinogenesis of all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9cRA), two metabolically active ROL derivatives (7). ATRA inhibited hepatic preneoplastic development and cell proliferation in rats initiated with N-nitrosomorpholine (8). Rats treated with ATRA or 9cRA during the progression phase of RH presented reduced HCC cell proliferation (9). On the other hand, ATRA failed to inhibit preneoplastic lesions (PNL) during the initial steps of hepatocarcinogenesis induced in rats with DEN (diethylnitrosamine) (10). ATRA induced hepatic cell proliferation in normal mice suggesting caution in its use for liver cancer control (11). In rat hepatocarcinogenesis ATRA chemopreventive activity was less effective than that of βC (1).

There is no reference in the literature to a study in which ATRA, 9cRA, ROL and βC chemopreventive activities were simultaneously compared during hepatocarcinogenesis. The objective of this study was to evaluate the chemopreventive effect of these ROL analogs during the initiation and progression phases of chemical hepatocarcinogenesis in rats submitted to the RH model. Hepatic PNL (in the form of foci/nodules) was induced by a single dose of diethylnitrosamine (DEN), followed by administration of 2-acetylaminofluorene (2-AAF) associated with a partial hepatectomy (6). Evaluated parameters included hepatic PNL development, cell proliferation, apoptosis and DNA damage.

A typical and critical property of hepatocyte foci and nodules induced by the RH model is their ability 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%) (12). Persistence of nodules could indicate a block in remodeling by differentiation (12) or apoptosis (13) and appears to be linked to enhanced evolution of HCCs (14).

Abbreviations: 2-AAF, 2-acetylaminofluorene; AI, apoptosis index; ATRA, all-trans retinoic acid; βC, β-carotene; CO, corn oil; DMSO, dimethylsulfoxide; GST-P, placent al glutathione S-transferase; HCC, hepatocellular carcinoma; H&E, hematoxylin–eosin; LI, labeling index; N, normal; 9cRA, 9-cis retinoic acid; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PH, partial hepatectomy; PNL, preneoplastic lesions; RH, resistant hepatocyte; ROL, retinol.
When administered specifically during the promotion phase of the RH model, βC chemopreventive activity involved stimulation of γ-glutamyltranspeptidase positive PNL remodeling (15). Thus, the ability of AtRA, 9cRA, ROL and βC to present similar actions on GST-P positive PNL remodeling was also investigated in the present study.

Materials and methods

Chemicals

9cRA was kindly provided by Kuraray (Osaka, Japan). AtRA, βC (trans-β-carotene type I), DEN, 2-AAF, dimethylsulfoxide (DMSO), 3,3'-diaminobenzidine, formaldehyde and low melting point agarose were purchased from Sigma (St Louis, USA). ROL (retinyl acetate) was purchased from Merck (Darmstadt, Germany). The commercial diet was purchased from Purina (Campinas, Brazil). Corn oil (CO) was Mazola® (São Paulo, Brazil). Polyclonal anti-GST-P rabbit antibody was purchased from Medical and Biological Laboratories (Tokyo, Japan). Polyclonal anti-proliferating cell nuclear antigen (PCNA) rat antibody, secondary biotinylated antibody and streptavidin-biotin-peroxidase complex (StrepABCPlx/HRP Duet, Mouse/ Rabbit) were purchased from Dako (Glostrup, Denmark). Other chemicals were of the highest available quality.

Animals and experimental protocol

Male Wistar rats from the colony of the Faculty of Pharmaceutical Sciences, initially weighing 50 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.

Figure 1 illustrates the experimental protocol design. At the end of a 7-day acclimatization period, with the exception of six Wistar rats not submitted to any experimental procedure [normal (N) group], 60 animals were randomly divided into 5 experimental groups. Those of the AtRA, 9cRA and ROL groups were treated with 1 mg/100 g body wt AtRA, 9cRA and ROL, respectively, and those of βC group were treated with 7 mg/100 g body wt βC. As all ROL analogs were dissolved in CO (0.25 ml/100 g body wt), animals receiving only CO (0.25 ml/100 g body wt) were used as controls (CO group). All treatments were performed by gavage, on alternate days for eight consecutive weeks. Rats were submitted to the RH model of hepatocarcinogenesis as follows (6): hepatocyte initiation was obtained by the administration of a single intraperitoneal dose of DEN (20 mg/100 g body wt) dissolved in 0.9% NaCl solution; after a recovery period of 2 weeks, the initiated hepatocytes were selected/promoted by the administration by gavage on consecutive days of four single doses of 2-AAF (3 mg/100 g body wt) dissolved in CO; 24 h after the last 2-AAF administration the animals were submitted to a potent mitogenic stimulus represented by a partial (2/3) hepatectomy (PH); finally, 2 and 4 days after PH, two additional AAF doses (2 mg/100 g body wt) were administered. All animals were euthanized by light ether anesthesia and exsanguination 6 weeks after DEN administration. The study was conducted in accordance with NIH guidelines for the care and use of laboratory animals.

Visible hepatocyte nodules counting

After the sacrifice the liver was removed from each animal, weighed and examined grossly on the surface and in 3-mm cross-sections for the presence of visible hepatocyte nodules of varied sizes and a generally whitish or yellowish color, different from the hepatic parenchyma.

Immunohistochemistry for GST-P and PCNA

Representative fragments of each liver lobe were fixed in methacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for ~24 h and included in paraffin. Immunohistochemical reaction of 5 μm sections were also processed in order to detect PNL (foci/nodules) positive for GST-P and hepatocytes positive for PCNA, according to the method described by Hsu et al. (16). After removal of paraffin, the endogenous peroxidase was blocked by 3% hydrogen peroxide in phosphate buffered saline (PBS) for 5 min. Thereafter, the sections were incubated overnight at 4°C with primary anti-GST-P rabbit or primary anti-PCNA rat antibody at a 1:1000 dilution in 1% bovine serum albumin (BSA). Finally, the sections were incubated for 1 h with secondary biotinylated antibody, thereafter the streptavidin-biotin-peroxidase complex was applied. Peroxidase binding sites were detected by incubation with 3,3′-diaminobenzidine (0.5%) and hydrogen peroxide (0.1%) dissolved in PBS, for ~2 min at room temperature. Sections were counterstained with hematoxylin.
Hepatocyte foci and nodules staining uniformly and non-uniformly for GST-P were classified as the persistent and remodeling type, respectively (17) (Figure 2). They were measured by the KS-300 program (Kontron Elektron, Munich, Germany) using a Nikon (Microphot-FXA, Tokyo, Japan) photomicroscope connected to a computer. Data were expressed as total (persistent + remodeling), persistent or remodeling GST-P positive PNL, Persistent or remodeling GST-P positive PNL were also expressed as a percentage of total (persistent + remodeling) GST-P positive PNL.

In order to evaluate PCNA labeling index (LI) 100 hepatocytes were analyzed per animal, of which 500 in PNL (foci/nodules) areas and 500 in the surrounding normal tissue (18), using a light microscope (Carl Zeiss). AI was expressed as the number of hepatocytes and nodules areas and 500 in the surrounding normal tissue (18), using a light microscope (Carl Zeiss). AI was expressed as the number of hepatocytes and nodules areas and 500 in the surrounding normal tissue (18), using a light microscope (Carl Zeiss).

Hepatocyte DNA strand breakage was evaluated in liver samples previously stored at −80°C, using the comet assay essentially as described by Toledo et al. (20). The tissues were smoothly homogenized in PBS (2.7 mM KCl, 8.1 mM Na2PO4, 1.5 mM KH2PO4, 0.14 M NaCl; pH 8.0), under refrigeration, and filtered. The isolated cells were then immobilized in a low-melting agarose (Sigma) matrix on a glass slide. Thereafter, these cells were lysed in the following solution: TBE buffer [90 mM Tris; 90 mM H3BO3; 2 mM Na2EDTA, (pH 8.4) and 2.5% SDS], and submitted to electrophoresis in TBE buffer at 62 V during 2 min. The resulting comets were stained with silver nitrate according to the method described by Nadin et al. (21), where silver is used instead of ethidium bromide. Normal rat liver tissues, challenged or not with hydrogen peroxide (10% final concentration, 5 min at room temperature and sonication) were used as negative and positive controls, respectively (20,22).

Length of the comets was evaluated using the previously described image analysis system. One hundred nucleoids per animal were randomly analyzed (50 images per slide). Coded slides were scored blindly. The viability of the liver cells was indirectly determined by analyzing the cell images after electrophoresis (23,24). The comet image was considered to be from a non-viable cell when it presented a ‘cloudy’ appearance or a very small head and a tail like a balloon (necrotic or apoptotic cells). The viability of the cell suspension was considered acceptable when the frequency of such images was <2% (23,24).

**Statistical analysis**

The Sigma Stat 2.0 (Jandel, San Rafael, USA) program was used for the statistical analysis. Fisher’s exact test was used when indicated. One-way ANOVA and Student’s t-tests were used when the results presented a normal distribution and, in the case this did not occur, Kruskall-Wallis and Mann-Whitney tests were used. Level of significance of \( P < 0.05 \) was applied to all cases.

**Results**

**Body and liver weights and incidence and multiplicity of visible hepatocyte nodules**

Table I presents the data on body and liver weights and incidence and multiplicity of visible hepatocyte nodules of rats treated during eight consecutive weeks with AtRA (AtRA group), 9cRA (9cRA group), ROL (ROL), \( \beta C \) (\( \beta C \)) or only CO (CO group, controls) and submitted to the RH model of hepatocarcinogenesis. No statistically significant differences (\( P > 0.05 \)) were observed between the different experimental groups regarding the final body weights, absolute and relative liver weights. These results indicate that these ROL analogs did not present toxicity at the doses used. When compared with the controls (CO group), animals of AtRA did not present differences (\( P > 0.05 \)) regarding incidence and multiplicity of hepatocyte nodules. Also compared with controls, 9cRA, ROL and \( \beta C \) groups presented lower multiplicity (\( P < 0.05 \)) of hepatocyte nodules, while only the carotenoid-treated group presented a lower incidence (\( P < 0.05 \)) of these PNL.

**Morphometric quantification of GST-P positive PNL**

GST-P is a marker of foci and nodules that can demonstrate a greater number and larger sizes of the putative PNL than other markers in various rat liver carcinogenesis studies (25). GST-P can also differentiate the persistent PNL (uniformly stained) from the remodeling ones (non-uniformly stained) (17). Table II shows the values obtained by morphometric quantification of the number and mean area of total

| Table I. Body and liver weights and quantification of visible hepatocyte nodules of rats treated with CO, AtRA, 9cRA, ROL or \( \beta C \) and submitted to the RH model of hepatocarcinogenesis |
|-------------------------------------------------|-----------------|----------------|-----------------|-----------------|
| Groups | Number of animals | Final weight (g) | Liver weight (g) | Relative liver weight (g/100 g body wt) | Incidence of nodules (%) | Multiplicity of nodules* |
|-------------------------------------------------|-----------------|----------------|-----------------|-----------------|
| CO | 12 | 195.3 ± 7.8 | 7.9 ± 0.5 | 3.9 ± 0.2 | 100 | 44 ± 9 |
| AtRA | 12 | 188.3 ± 10.1 | 6.7 ± 0.3 | 3.6 ± 0.1 | 92 | 27 ± 9 |
| 9cRA | 12 | 204.2 ± 7.4 | 7.3 ± 0.2 | 3.5 ± 0.1 | 92 | 11 ± 4b |
| ROL | 11 | 205.1 ± 9.0 | 7.2 ± 0.3 | 3.6 ± 0.3 | 82 | 7 ± 3b |
| \( \beta C \) | 11 | 189.1 ± 19.5 | 7.3 ± 0.2 | 3.9 ± 0.5 | 46 | 4 ± 2b |

Values are means ± SEM (except for incidence of nodules).
*Multiplication: mean number of nodules/rat presenting nodules.
Statistically significant difference compared with the CO group (Mann–Whitney’s test, \( P < 0.05 \)).
Statistically significant difference compared with the CO group (Fisher’s exact test, \( P < 0.05 \)).
AtRA, 9cRA, ROL and βC groups presented a lower number of persistent or remodeling GST-P positive PNL when compared with the CO group, but not all other ROL analog-treated groups, presented smaller (P < 0.05) persistent GST-P positive PNL when compared with the CO group. AtRA, 9cRA, ROL and βC groups presented total (persistent + remodeling) persistent or remodeling GST-P positive PNL that occupied a smaller (P < 0.05) area of the liver section when compared with the CO group.

Cell proliferation and apoptosis

Figure 3 shows PCNA LI of areas surrounding PNL and of the PNL areas themselves at the end of the 8 weeks of experiment. In all experimental groups PCNA LI of PNL areas was higher (P < 0.05) than PCNA LI of the respective surrounding normal tissue areas. These results agree with the information that during hepatocarcinogenesis cell proliferation increases (26). Compared with the CO group, AtRA, 9cRA, ROL and βC groups presented smaller PCNA LI in normal tissue areas surrounding PNL (P < 0.05) and in PNL areas (P < 0.05).

Hepatic DNA strand breakage

In this study we opted for staining the formed comets with silver nitrate because of the advantages of the method, for example, to allow the permanent record of the experiment and independent verification of the results, as well as to avoid problems associated with fluorescence such as decay. In addition, staining with silver allows the comets to be analyzed using a simple light microscope instead of expensive and complex equipment such as fluorescence microscopes (20,22).

Values are means ± SEM.

**Table II.** Morphometric analysis of GST-P positive PNL of rats treated with CO, AtRA, 9cRA, ROL or βC and submitted to the RH model of hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Number of GST-P positive PNL per cm²</th>
<th>Percent remodeling GST-P positive PNL*</th>
<th>Size of GST-P positive PNL (mm²)</th>
<th>Area of liver section occupied by GST-P positive PNL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>P</td>
<td>R</td>
<td>T</td>
</tr>
<tr>
<td>CO</td>
<td>12</td>
<td>166 ± 14</td>
<td>23 ± 3</td>
<td>143 ± 12</td>
<td>0.18 ± 0.09</td>
</tr>
<tr>
<td>AtRA</td>
<td>12</td>
<td>107 ± 13a</td>
<td>11 ± 2a</td>
<td>96 ± 12a</td>
<td>0.12 ± 0.06‡</td>
</tr>
<tr>
<td>9cRA</td>
<td>12</td>
<td>71 ± 12a</td>
<td>7 ± 2a</td>
<td>63 ± 11a</td>
<td>0.12 ± 0.06‡</td>
</tr>
<tr>
<td>ROL</td>
<td>11</td>
<td>96 ± 13a</td>
<td>4 ± 1a</td>
<td>92 ± 12a</td>
<td>0.11 ± 0.22ª</td>
</tr>
<tr>
<td>βC</td>
<td>11</td>
<td>106 ± 13b</td>
<td>6 ± 1b</td>
<td>100 ± 2a</td>
<td>0.08 ± 0.03a</td>
</tr>
</tbody>
</table>

Fig. 3. Quantification of PCNA LI of rats treated with CO, AtRA, 9cRA, ROL or βC and submitted to the RH model of hepatocarcinogenesis. Values are means ± SEM, n = 12 (CO, AtRA and 9cRA groups) and 11 (ROL and βC groups). Statistics by Mann–Whitney’s test: Statistically significant differences (P < 0.05), a: when compared with the respective surrounding normal tissue area, b: when compared with the CO group (controls) preneoplastic lesions area and c: when compared with the CO group (controls) preneoplastic lesions area.

Fig. 4. Quantification of apoptosis index of rats treated with CO, AtRA, 9cRA, ROL or βC and submitted to the RH model of hepatocarcinogenesis. Values are means ± SEM, n = 12 (CO, AtRA and 9cRA groups) and 11 (ROL and βC groups). Statistics by Mann–Whitney’s test: Statistically significant differences (P < 0.05), a: when compared with the respective surrounding normal tissue area.
Table III. Lengths of comets of normal rat livers challenged\(^*\) or not\(^b\) with hydrogen peroxide (HP), as well as of livers of rats treated with CO, AtRA, 9cRA, ROL or βC and submitted to the RH model of hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Comet length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>81.8 ± 2.9</td>
</tr>
<tr>
<td>N(HP)</td>
<td>6</td>
<td>187.7 ± 8.2(^c)</td>
</tr>
<tr>
<td>CO</td>
<td>12</td>
<td>100.4 ± 3.9(^c)</td>
</tr>
<tr>
<td>AtRA</td>
<td>12</td>
<td>94.8 ± 4.1</td>
</tr>
<tr>
<td>9cRA</td>
<td>12</td>
<td>92.4 ± 1.5</td>
</tr>
<tr>
<td>ROL</td>
<td>11</td>
<td>87.9 ± 2.6(^d)</td>
</tr>
<tr>
<td>βC</td>
<td>11</td>
<td>89.2 ± 4.0(^d)</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

\(^*\)Positive comet assay controls.

\(^b\)Negative comet assay controls.

\(^c\)Statistically significant difference compared with the N group (Student’s \(t\)-test, \(P < 0.05\)).

\(^d\)Statistically significant difference compared with the CO group (Student’s \(t\)-test, \(P < 0.05\)).

Discussion

Medical treatment for HCC remains elusive and procedures to prevent the disease are urgently desired. One such approach is the current use of acyclic retinoid for chemoprevention of a second primary liver tumor (2), based on previous observations that the compound induces apoptosis of human hepatoma-derived cell lines and inhibits experimental liver carcinogenesis (28).

There are several models for the study of liver cancer development with chemicals. In many of these models the growth of the hepatocytes in foci is very slow and generally asynchronous, taking many weeks or even a few months to form visible hepatocyte nodules (12). In the RH model this is accomplished in a rapid and a synchronous fashion following initiation with DEN and selection/promotion with 2-AAF and PH, so that nodules are seen grossly within a week or 10 days after the selection pressure is applied. The RH model is based on the concept that 2-AAF generates a differential mitoinhibitory effect on the non-initiated hepatocytes, while permitting the initiated hepatocytes (the resistant ones) to respond to liver cell proliferative stimulus such as that induced by PH (14). Among the various protocols of rat hepatocarcinogenesis, the RH model is well characterized and is particularly adapted to compare effects of compounds potentially able to modulate an ongoing carcinogenic process (29).

Studies of the effects of ROL and its natural analogs on hepatocarcinogenesis have been equivocal. Suppression and enhancement by these substances were reported in different models of liver carcinogenesis (30). In the present study, based on data of the macroscopic analysis of visible hepatic nodules and of morphometry of GST-P positive PNL, similar chemopreventive activities were shown following treatment of rats with AtRA, 9cRA, ROL or βC during the initiation and promotion phases of the RH model. This indicates that these natural ROL analogs represent promising chemopreventive agents against hepatocarcinogenesis.

One of the most positive features of the RH model is its ability to distinguish the few persistent nodules from the large number of remodeling ones and thus to allow studies of the nodule to cancer sequence (14). Interestingly, in the present study AtRA, 9cRA, ROL and βC inhibited the development of persistent GST-P positive PNL, considered to be precursors of HCC (14,31). Moreover, when remodeling GST-P positive PNL are expressed as a percentage of total (persistent + remodeling) GST-P positive PNL, there is an increase in remodeling in 9cRA, ROL and βC groups compared with the CO group. Similar results were previously observed during chemoprevention with βC when it was specifically administered during the promotion phase of the RH model (15). Chemoprevention by \(S\)-adenosyl-L-methionine (SAM) in the RH model also involves stimulation of PNL remodeling (32). On the other hand, in the present study AtRA did not increase the percentage of remodeling PNL. This was also described during chemoprevention of hepatocarcinogenesis by the synthetic retinoid N-(4-hydroxyphenyl)retinamide (33). Thus, increased remodeling represents an important mechanism by which 9cRA, ROL and βC, but not AtRA, could exert their chemopreventive effects during hepatocarcinogenesis. Remodeling has also been described during colon (34) and mammary gland (35) carcinogenesis. It would be interesting to evaluate if these ROL analogs could also increase remodeling of aberrant crypt foci and mammary gland intraductal proliferations.

AtRA, 9cRA, ROL and βC inhibit the proliferation of several lineages of neoplastic cells in culture (36,37). However, only few studies investigated the \textit{in vivo} action of these substances on cell proliferation, especially in hepatocarcinogenesis models (9). AtRA, 9cRA, ROL and βC strongly inhibited cell proliferation in hepatic neoplastic lesions during the progression phase of the RH model (5,9). Similar effects on PNL cell proliferation have now been demonstrated in the present study following treatment with these ROL analogs during the initiation and promotion phases of this model. Thus, AtRA, 9cRA, ROL and βC chemopreventive activities could be attributed to inhibitory effects on cell proliferation. On the other hand, apoptosis induction by these substances was not observed in the present study, suggesting that this mechanism is not involved in their chemopreventive activities.

Triiodothyronine (T3)-induced hepatic nodule loss appears to be related to remodeling by redifferentiation, but not by apoptosis induction (38). Inhibitory effects of dehydroepiandrosterone or 16α-fluoro-5-adrosten-17-one during the promotion phase of the RH model involved increased percentage of remodeling GST-P positive PNL and reduced cell proliferation in these specific PNL (39). Hepatic PNL remodeling is under genetic control (12,40) and cell cycle
and differentiation-related genes seem to be involved (41). However, the exact genes responsible for remodeling are unknown (40). Downregulation of c-myc and cyclins and upregulation of \( p16^{INK4a} \) have been suggested to occur during remodeling (41). Thus, in further experiments it would be interesting to evaluate during chemoprevention of hepatocarcinogenesis by 9cRA, ROL and βC cell proliferation and differential gene expression specifically in remodeling and persistent PNL.

Oxidative stress has an important role during initiation, promotion and progression of hepatocarcinogenesis (42). Thus, the importance of oxidative damage to DNA in hepatocarcinogenesis should be emphasized (43).

In the present study ROL and βC, but not ATRA and 9cRA, inhibited hepatic DNA damage. Moreover, the retinoic acids did not induce DNA damage as measured by the comet assay. This method enables the evaluation of ROS-induced oxidation and DNA strand breaks, (44). ROL and its natural analogs constitute potential cancer chemopreventive agents with varied antioxidant activity that is related to their hydrophobic lateral polyenic chain (45). Therefore, ROL and βC inhibitory effects on DNA damage during the initial phases of hepatocarcinogenesis could be related to their antioxidant potential. This suggestion is reinforced by the observation that lutein and lycopene, carotenoids that present high antioxidant potential, were able to inhibit DNA damage as measured by the comet assay also in rats submitted to the RH model (20).

Altogether, the results of the present study indicate that ATRA, 9cRA, ROL and βC represent promising chemopreventive agents against hepatocarcinogenesis. Furthermore, inhibition of cell proliferation, but not induction of apoptosis, is important for anticarcinogenic actions of all substances. Increased remodeling of GST-P positive PNL relates to 9cRA, ROL and βC actions, while inhibition of DNA damage relates to ROL and βC actions.

Supplementary material

Supplementary material can be found at: http://carcin.oxfordjournals.org/

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