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Effects of β-carotene and vitamin A on oval cell proliferation and connexin 43 expression during hepatic differentiation in the rat

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Abstract

The effects of β-carotene and vitamin A administrations were evaluated in an in vivo model of hepatic cell differentiation. For this purpose, male Wistar rats received β-carotene (70 mg/kg of body weight), vitamin A (10 mg/kg of body weight) or corn oil (control group), by gavage and at every other day during the entire experimental period. After 4 consecutive weeks of treatment, the animals were submitted to the AAF/PH model of hepatic cell differentiation (6 × 20 mg of AAF [2-acetylaminofluorene]/kg of body weight and partial hepatectomy) and killed on different days following the surgery (until day 16 after hepatectomy). Liver samples were collected for determination of β-carotene, retinol and retinyl palmitate concentrations, for histopathological (hematoxilin-eosin) examination, for immunohistochemical detection of glutathione S-transferase, as well as for the evaluation of connexin 43 (a structural protein of gap junctions of oval cells) expression by northern blot analysis. Compared to controls, the oval cell proliferation peaks (observed by histopathological examination and immunohistochemistry) and connexin 43 expression peaks, were postponed to later days after hepatectomy, in a similar way in β-carotene and vitamin A treated animals. Compared to the other experimental groups, the vitamin A treated group showed an increase in connexin 43 expression. It was concluded that β-carotene and vitamin A modulated oval cell proliferation and connexin 43 expression, delaying both events. These findings suggest that β-carotene and vitamin A can modulate the hepatic differentiation process in vivo.

Keywords: Hepatic differentiation; Oval cell; Connexin 43; β-carotene; Vitamin A

1. Introduction

The epidemiological evidences that support a role of β-carotene in cancer chemoprevention have been especially confirmed in either *in vitro* or *in vivo* experimental studies [1,2]. In the latter case, an inhibitory activity of the carotenoid was observed in hepatocarcinogenesis models such as the resistant hepatocyte, among others [3–8].

In *in vitro* models such as the C3H10T1/2 cell system of mouse fibroblasts, β-carotene has shown to be an agent capable to inhibit the neoplastic transformation [1]. This effect has been reported to be partially mediated through the induction of gap junctional intercellular communications, due to the ability of the carotenoid to modulate the expression of the gene that codifies for connexin 43 (cx 43), a structural protein of gap junctions in C3H10T1/2 cells [9–12]. Gap junctional intercellular communications consist in an important mechanism by which cells exchange hydrophilic molecules with low molecular weight to control differentiation and proliferation, and which are able to inhibit or even to suppress the carcinogenic process [13–16]. Cancer, in the meantime, is characterized by the production of clones of terminally non-differentiated or "dedifferentiated" cells that maintain their immortality and capability of reacting to proliferative stimulus [17].

It has been postulated that activated progenitor cells

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(stem cells) in the liver of an adult animal can generate the so called oval cells, small cells with oval nuclei seen around portal spaces, which proliferate during the initial phases of hepatocarcinogenesis and can differentiate into hepatocytes and into biliary epithelial cells [18]. Thus, it was demonstrated that the differentiation of oval cells into hepatocytes is followed by a switch from connexin 43 (of oval cells) expression to connexins 32 and 26 (of hepatocytes) expressions in the liver of rats [19]. Furthermore, oval cells can originate hepatic cancers, by an irreversible block in the process of normal differentiation [18,20].

It has been demonstrated, in neoplastic cell lines in culture, that β-carotene (and other carotenoids without provitamin A activity) induces cell differentiation [21,22]. On the other hand, it was observed, in the resistant hepatocyte model of hepatocarcinogenesis, that β-carotene reduced the number of oval cells at the end of the experimental period [3–5,23]. This finding may be an evidence of the action of the carotenoid in the hepatic cell differentiation process, which could explain its chemopreventive activity against preneoplastic lesions of the liver [3–8].

Therefore, in order to contribute to a better understanding of the eventual role of β-carotene in cell differentiation and, consequently, in chemoprevention of cancer, the main objective of the present study was to investigate the effects of the administration of the carotenoid or of vitamin A to rats submitted to a hepatic cell differentiation model, on the process of oval cell proliferation and of connexin 43 expression.

2. Material and methods

2.1. Animals and experimental design

Male Wistar rats (50–70g) from the colony of the Faculty of Pharmaceutical Sciences of the University of São Paulo (São Paulo, Brazil) were maintained under standard environmental conditions. The animals had free access to industrialized food (Purina Nutrimentos Ltda., Campinas, SP, Brazil) and were treated with β-carotene (BC group, 70 mg/kg of body weight, *trans*-beta carotene type I, Sigma), vitamin A (VA group, 10 mg/kg of body weight, retinyl acetate, Sigma), or corn oil (CO group—controls), by gavage, every other day and during the entire experimental period. After 4 consecutive weeks of treatment, the animals (with approximately 200 g of body weight) were submitted to the AAF/PH model of oval cell differentiation [24], adapted to Wistar rats (4 + 2 doses [20 mg/kg of body weight, administered by gavage] of AAF [Sigma, dissolved in corn oil] respectively before and after a 70% partial hepatectomy) (Fig. 1). The AAF/PH model consists in an uninitiated version (without diethylnitrosamine administration as an initiating agent) of the modified resistant hepatocyte model of hepatocarcinogenesis [25], and in which 9 doses of AAF (1 mg per dose) are administered to F-344 rats. In the present study, considering that Wistar rats are more resistant to the action of AAF than F-344 animals [26], doses of AAF similar to the ones (6 × 20 mg/kg of body weight) usually employed in the resistant hepatocyte model adapted to Wistar rats [4,5,23] were utilized.

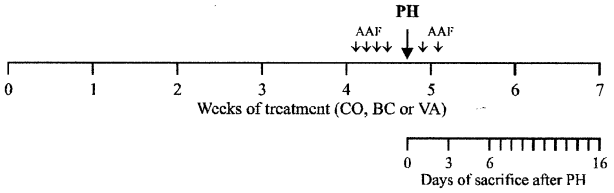

Fig. 1. Schematic representation of experimental design. PH, partial hepatectomy (70%). AAF, 2-acetylaminofluorene (6 × 20 mg/kg of body weight). CO, corn oil. BC, beta-carotene (70 mg/kg of body weight). VA, vitamin A (10 mg/kg of body weight).

The rats were killed (4 rats/day/treatment) by exsanguination under ether anesthesia on days 0, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 following partial hepatectomy (Fig. 1). At the sacrifice, the liver samples collected for the determination of β-carotene and vitamin A concentrations and for RNA evaluation were quickly frozen in liquid nitrogen and stored at −70°C.

2.2. Determination of β-carotene and vitamin A hepatic concentrations

The hepatic concentrations of BC and VA (retinol and retinyl palmitate) were determined by reverse phase HPLC (high performance liquid chromatography) [27] using a Shimadzu LC9A chromatographer with a multisolvent pumping system, an auto-injector (SIL-6B) and a photodiode array UV-VIS detector (SPD-M6A) (adjusted at 450 nm for the detection of β-carotene and at 325 nm for the detection of retinoids). Chromatography was carried out on a C-18 reversed phase column (CLC-ODS; 5μm, 6mm ID × 15 cm) protected by a guard column (CLCG-ODS). The substances were eluted (flow rate at 1,5 ml/min) using the same mobile phase composed by a mixture of acetonitrile, dichloromethane and methanol (20:20:10). VA (retinol and retinyl palmitate) and BC were identified by comparison of their retention times (β-carotene = 12.5 min, retinol = 2.9 min and retinyl palmitate = 13.1 min) with those of authentic standards, and quantified by peak areas.

2.3. Histopathological examination and immunohistochemistry

Representative fragments of each hepatic lobe were fixed in methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid, P.A.) and embedded in paraffin, in order to obtain serial sections of approximately 5 μm. The sections were stained with hematoxilin-eosin (H&E) for morphological examination (presence of oval cells, neo-created ducts and hepatocyte foci). Anti-GST-P (placental glutathi-

one S-transferase) antibodies were utilized to staining of oval cells by immunohistochemistry [23,28]. The slices were incubated with primary antibodies (1:4.000; rabbit anti-GST-P polyclonal antibodies, MBL, Japan) and with anti-mice/rabbit immunoglobulin secondary biotinilated antibodies (Strept ABComplex/HPR, Duet, mouse/rabbit, DAKO KO492, Denmark), followed by the application of streptavidin–biotin-peroxidase complex and detection with diaminobenzidine, according to the method described by Hsu et al. [29].

The oval cells were quantified by the technique of volume estimation through intersection points, adapted from Weibel [30]. Fifteen periportal zones considered as areas of ductular cell proliferation were analyzed in each slice, in every other portal spaces of the right anterior lobe section. The points of intersection with GST-P positive periportal structures (oval cells) were recorded, and the results were expressed in cell volume fraction (%) of periportal zones, as described by Dagli et al. [23].

2.4. Northern blot analysis

The expression of the gene that codifies for connexin 43 was evaluated by northern blot analysis. For this purpose, total RNA was isolated from approximately 500 mg of liver tissue by acid guanidinium thiocyanate-phenol-chloroform extraction [31], separated by electrophoresis, transferred to nitrocellulose membranes (Sigma, W-8248) and hybridized, in accordance with Sambrook et al. [32]. The cDNA probe of cx 43 (inserted in BSKS+ plasmid) was kindly provided by Dr. David L. Paul (Department of Neurobiology, Harvard Medical School, Boston, USA) and labeled with ^{32}P-dCTP (Amersham AA0075). A DNA solution (Herring Sperm DNA, Gibco 15634−017) was used during the hybridization procedure, which was performed in a hybridization oven at a constant temperature of 42°C for approximately 48 h. Autoradiographies were carried out at −70°C during periods varying between 2 to 4 days, using Kodak X-OMAT films in X-ray cassettes (Kodak/Sigma E-9010) fitted with intensifying screens.

Quantitative densitometry of the autoradiograms was performed using a Bio-Rad Imaging Densitometer (Model GS-700), with the aid of the Molecular Analyst software (Bio-Rad's Image Analysis Systems, 1.4 version).

Relative mRNA levels were normalized for loading variability by comparison with β-actin mRNA levels in the same filters (cDNA probe; Oncor P 7000, USA).

2.5. Statistical analysis

When indicated, the statistical analysis of data was done by ANOVA and the Student's *t* test for unpaired samples [33], using the INSTAT software (GraphPad Software, 2.01 version, copyright 1990–1992) for the respective calculations.

Table 1
β-Carotene, retinol and retinyl palmitate concentrations (μg/g of tissue)[a] per treatment and days of sacrifice after partial hepatectomy (PH), in the livers of Wistar rats treated by gavage every other day with corn oil (CO-control), β-carotene (BC) or vitamin A (VA) and submitted to the AAF/PH model of hepatic cell differentiation

Days of Sacrifice After PH	Group		
	CO	BC	VA
β-Carotene			
Zero	0.10 ± 0.03	2.54 ± 0.72**††	0.12 ± 0.06
Day 16	0.11 ± 0.04	2.58 ± 0.76**††	0.16 ± 0.05
Retinol			
Zero	9.90 ± 3.74	21.09 ± 6.34*††	72.20 ± 24.04**
Day 16	12.50 ± 3.70	21.89 ± 3.52**††	71.80 ± 21.82**
Retinyl Palmitate			
Zero	12.81 ± 6.15	32.06 ± 12.39*††	138.17 ± 31.54**
Day 16	14.19 ± 3.77	24.80 ± 4.13**†	117.01 ± 58.14**

[a] Values are means of 4 rats ± SD. Difference statistically significant (unpaired Student's *t* test) compared to the CO group (* = $P < 0.05$; ** = $P < 0.01$) and to the VA group († = P 0.05; †† = $P < 0.01$).

3. Results

In the present study, treatment with β-carotene (BC group), at the dose of 70 mg/kg of body weight every other day and during 5–7 consecutive weeks, resulted in hepatic concentrations of carotenoids that were significantly higher ($P < 0.01$) than those of the other experimental groups (about 20 times higher than the CO group and 10 times higher than the VA group) (Table 1). Furthermore, in agreement with previous observations in the resistant hepatocyte model of hepatocarcinogenesis [5], a significant increase in the hepatic levels of retinol and retinyl palmitate was observed in the BC group, in comparison to the CO group. In addition, as expected, the VA group presented higher hepatic concentrations of vitamin A (retinol and retinyl palmitate) compared to the two other experimental groups (Table 1).

The kinetics of oval cell proliferation, observed by H&E (Fig. 2), was confirmed by the morphometric quantification of GST-P positive oval cells (Fig. 3). Thus, it was observed that on day 9 post-PH, the CO group presented an oval cell

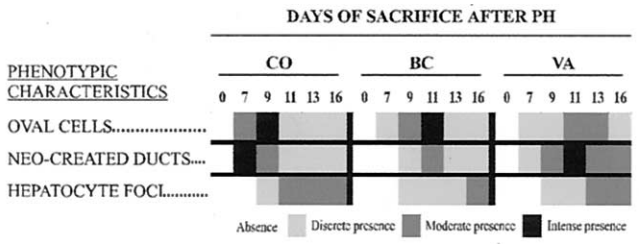

Fig. 2. Schematic representation of the results from histopathological analyses (H&E) in the livers of rats treated by gavage with corn oil (CO-control), β-carotene (BC) or vitamin A (VA), submitted to the AAF/PH model of oval cell differentiation and sacrificed on different days after partial hepatectomy (PH). The data are representative of 4 animals.

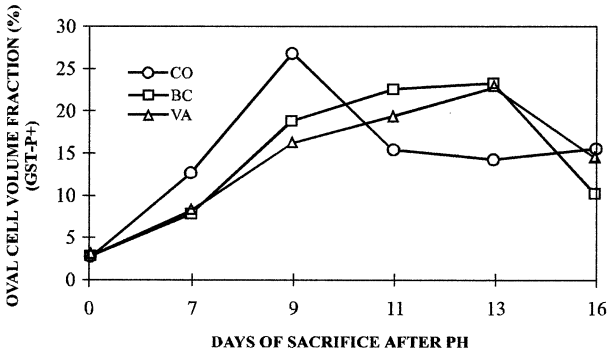

Fig. 3. Volume fraction (%) of the area occupied by GST-P positive oval cells, in periportal regions of the livers of Wistar rats (mean of 3 or 4 animals) treated by gavage with corn oil (CO- control), β-carotene (BC) or vitamin A (VA), submitted to the AAF/PH model of oval cell differentiation, and sacrificed on different days after partial hepatectomy (PH).

proliferation significantly higher ($P < 0.01$) than the BC and VA groups (respectively 26.67 ± 1.53%, 18.67 ± 0.58% and 16.25 ± 3.30%). Fig. 4 presents photomicrographs that illustrate these analyses. On day 11 post-PH, the volume fraction occupied by the oval cells was reduced in the CO group and kept the same proportions on the subsequent days (13 and 16). On the contrary, an intense presence of oval cells was observed in the BC and VA groups between days 11 and 13 post-PH, which was similar in the BC and VA groups and significantly higher ($P < 0.05$) than the one present in the CO group on day 13 post-PH (23.00 ± 3.00%, 22.67 ± 4.93%, 14.00 ± 2.94%, respectively). On day 16 post-PH, the number of oval cells observed in the periportal zones in the CO and VA groups tended to be higher than in the BC group, i.e., 15.25 ± 3,86%, 14,25 ± 3,30% and 10.00 ± 2,45%, respectively, although the differences did not reach statistical significances ($P > 0.05$).

The kinetics of connexin 43 expression of the three experimental groups is illustrated in the autoradiograms presented in Fig. 5. It was observed that cx 43 (of oval cells)

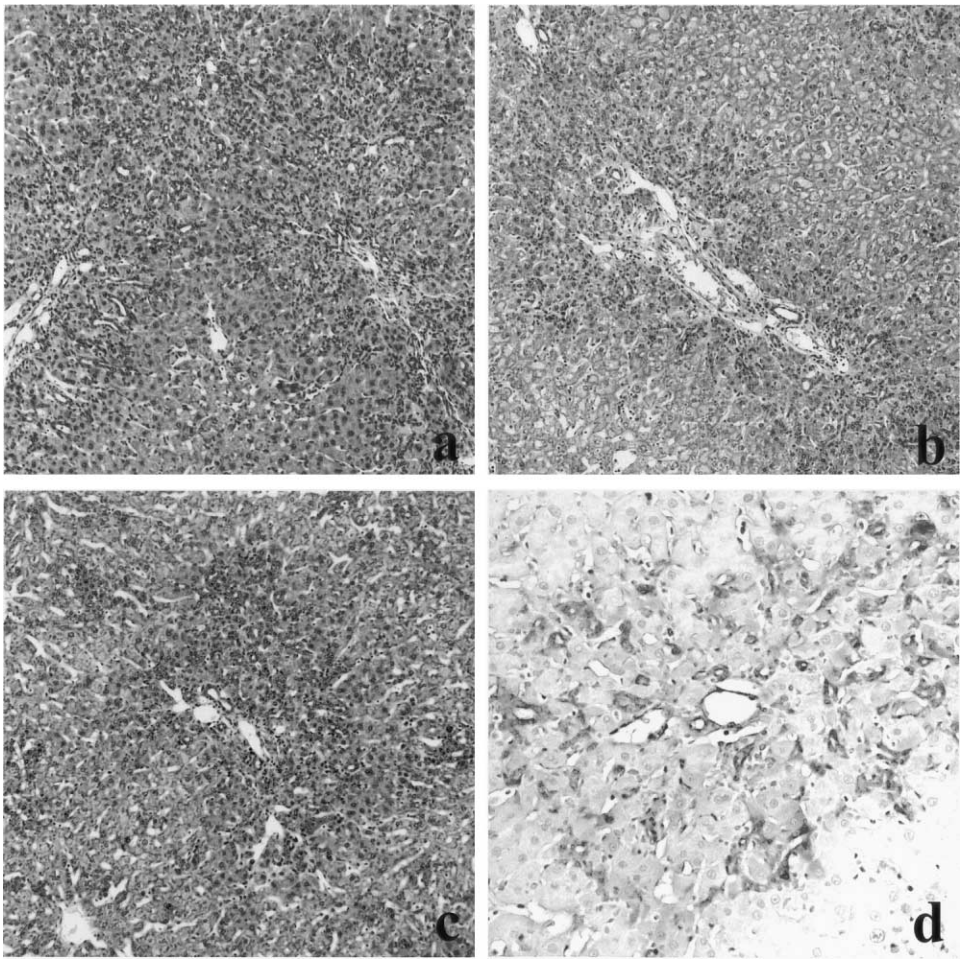

Fig. 4. Histopathological alterations in the livers of rats treated with corn oil, vitamin A or β-carotene and submitted to the AAF/PH model of oval cell differentiation and sacrificed on day 9 after partial hepatectomy. a. Liver section of a CO group animal, showing oval cells intensely proliferating from the portal spaces and invading the liver parenchyma (H&E, Obj. 10×); b. Liver section of a VA group animal, showing less oval cell proliferation from the portal space than in the animal of the CO group (H&E, Obj. 10×); c. Liver section of a βC group animal, also showing less oval cells than in the animal of the CO group; d. Immunostaining of GSTP in oval cells in the liver of a rat treated with β-carotene (20×).

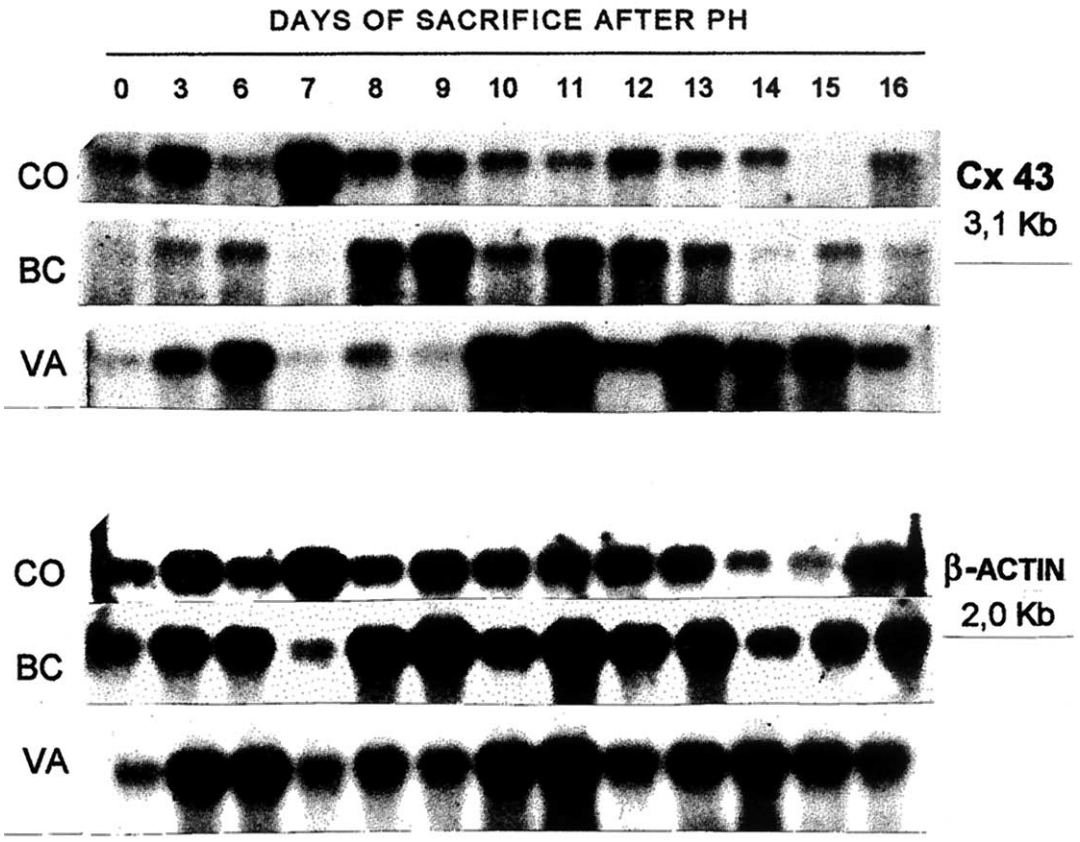

Fig. 5. Expression of the gene that codifies for connexin 43 (cx 43) in the livers of Wistar rats treated with corn oil (CO- control), β-carotene (BC) or vitamin A (VA), submitted to the AAF/PH model of oval cell differentiation and sacrificed on different days after partial hepatectomy (PH). Each autoradiograph illustrates the results of three experiments. β-Actin was used as a control.

expression was higher during the first days post-PH in the CO group (between days 3 and 8) and during the later days in the other two experimental groups, i.e., BC group showed a higher cx 43 expression between days 8 and 12 after the surgery, whereas the VA group showed a higher connexin 43 expression between days 10 and 15 post-PH. The results of the densitometric analysis of the autoradiograms, corrected by β-actin gene expression and relative to day-zero (at the hepatectomy), are shown in Fig. 6. It can be observed in this figure that the highest peak of cx 43 gene expression occurred on day 7 post-PH in the CO group and on later days in the BC and VA groups (days 11 and 12 post-PH, respectively). Moreover, vitamin A-treated animals presented the highest level of cx 43 expression in relation to day-zero (4.4 times higher on day 12 post-PH), when compared to the BC (2.5 times higher on day 11 post-PH) and CO groups (3.5 higher on day 7 post-PH).

4. Discussion

In agreement with previous reports [24,34,35], it was observed in the present study, either by H&E or immunohistochemistry for GST-P, that the animals of all experimental groups (BC, VA and CO groups) showed a remarkable presence of oval cells in their livers, which tended to invade the hepatic acinus from the periportal region. Moreover, the kinetics of oval cell proliferation (Figs. 2, 3 and 4) observed in the control group (CO group) was very similar to that reported by Evarts *et al.* [24] in F-344 rats. On the other hand, the administration of β-carotene or vitamin A to

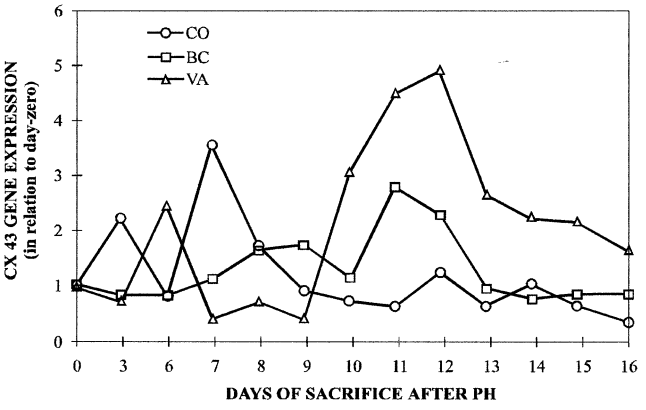

Fig. 6. Expression (in arbitrary units) of the gene that codifies for cx 43 (compared to day-zero) in the livers of Wistar rats treated with corn oil (CO- control), β-carotene (BC) or vitamin A (VA), submitted to the AAF/PH model of oval cell differentiation and sacrificed on different days after partial hepatectomy (PH).

the animals similarly delayed the oval cell proliferation in BC and VA groups, respectively (Fig. 2 and 3).

Studies have reported that the oval cells elicited by the AAF/PH model, which first appear in the periportal areas, could originate bile duct-like structures that diffuse into the hepatic parenchyma [18,36,37]. This is in agreement with observations of the present experiment (Fig. 2). Thus, it has been suggested that during the process of liver recovery, the new population of hepatocytes would be originated either from oval cells [24,34,38] or from epithelial cells of the small neo-created ducts [37,38]. Furthermore, these epithelial cells would constitute the progenies of oval cells [36].

It was observed in this study that the animals of the BC group showed a tendency to present lower amounts of neo-created bile ducts and hepatocyte foci (Fig. 2), as well as a lower cellularity in the periportal region on day 16 post-PH in their livers (Fig. 3), than rats of the two other experimental groups (VA and CO groups). These findings are consistent with results of earlier investigations conducted in rats submitted to a hepatocarcinogenesis model [3–5,23], in which the carotenoid showed inhibitory effects on preneoplastic lesions and reduced the number of oval cells at the end of the experimental period. In contrast to the results of the kinetics of oval cell proliferation presented previously and which indicated an action of β-carotene that could have been mediated via retinoids, these data indicate now an intrinsic action of the carotenoid that seems to be associated with the induction of oval cell differentiation and their intermediate progenies into hepatocytes. Indeed, the accumulation of β-carotene and the presence of considerable levels of vitamin A (retinol and retinyl palmitate) in the livers of the animals of the BC group (Table 1), reinforce the possibility of an action of the carotenoid either in its intrinsic form or in the form of active retinoids, mainly as retinoic acid (via an oxidative metabolism of retinol) [39, 40].

Since it has been demonstrated that β-carotene induces cell differentiation [21,22] and that oval cells can originate hepatic cancers [18,20], when examined altogether the data presented and discussed above indicate that the induction of oval cell differentiation could be one of the mechanisms eventually involved with the chemopreventive action of the carotenoid described on experimental hepatocarcinogenesis.

Similar effects on cx 43 expression (the structural protein of the gap junctions of oval cells) where observed in the present study in Wistar rats submitted to the AAF/PH model of hepatic cell differentiation and treated with β-carotene or vitamin A. Thus, compared to the control group (CO group) it was observed that the cx 43 expression curves related to both BC and VA groups showed a tendency to exhibit peaks of expression on later days post-PH. However, the levels of expression in the VA group in relation to the day of PH (day-zero) were higher than those of the BC group (Fig. 6).

Therefore, treatment with vitamin A under these *in vivo* experimental conditions resulted in an increase in cx 43 expression. This finding is in agreement with reports of the literature, which indicate that retinoids can increase cx 43 expression in *in vitro* models, either at the transcriptional or post-transcriptional level [41,42]. Thus, it has been described that retinoids could eventually induce homologous intercellular communications, not only between oval cells, but also between oval and Ito cells. Ito cells (also called perisinusoidal stellate cells), which express cx 43 and also constitute the main deposit of vitamin A in the liver, are known to follow the oval cell migration during hepatic regeneration as well as to secrete growth factors [35,43,44]. Therefore, these findings suggest that retinoids can enhance oval cell proliferation and differentiation.

In a similar way to retinoic acid, β-carotene has been reportedly capable to induce cx 43 expression in an *in vitro* model of mouse fibroblast cells, by transcriptional and/or post-transcriptional mechanisms [9–12]. At least part of this action could be mediated by active retinoids formed via metabolic (enzymatic) conversion [45] or during the oxidation of these compounds (chemical conversion) [46,47]. However, in the *in vivo* model used in the present experiment, treatment with β-carotene did not cause an increase in cx 43 expression of hepatic oval cells, when compared to the control group (Figs. 5 and 6).

It can be observed in the present investigation that the peaks of oval cell proliferation (Fig. 3) and the peaks of cx 43 expression (Fig. 6) presented a similar tendency, i.e., they occurred later in the the BC and VA groups (between days 11 and 13 post-PH) than in the control group (CO group- between days 7 and 9 post-PH). It should be stressed that in this study conducted in Wistar rats, the evolution of cx 43 expression observed in the BC and VA groups is consistent with previous reports [19,44]. However, the period of the greatest cx 43 expression in CO group occurred earlier than the one observed in F-344 rats submitted to the AAF/PH model of hepatic cell differentiation [19]. These differences may be eventually attributed to the different strains of rats used in both studies.

The discrepancy between the number of oval cells (Fig. 3) and the levels of cx 43 expression (Fig. 6) on day 16 post-PH, especially observed in the BC and CO groups of this investigation, does not reflect a direct relationship between cx 43 gene expression and the oval cell differentiation process, as described by Zhang and Thorgeirsson [19]. In this context, more detailed studies involving an eventual and direct action of β-carotene (and other carotenoids) and retinoids on the formation of gap junctional communications during the cell differentiation process in an *in vivo* model such as the AAF/PH, would be obviously required. Furthermore, it would be important to determine in an animal model, by immunohistochemistry and in situ hybridization studies whether carotenoids can specifically modulate the cellular distribution of cx 43 protein and transcripts in oval cells.

In conclusion, β-carotene and vitamin A modulated oval cell proliferation and cx 43 expression during hepatic dif-

ferentiation in the rat, delaying both events. These findings suggest that β-carotene and vitamin A can modulate the hepatic differentiation process *in vivo*.

Acknowledgments

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