The Role of Retinyl Acetate in Chemoprevention

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Abstract Retinoids have a role in the process of chemoprevention. They can inhibit promotion through inhibiting cell proliferation, enhancing apoptosis and anti-oxidative properties. In the present study initiative-p-dimethylaminoazobenzene (p-DAB) and promotive-2, 3, 7, 8-Tetrachlorodibenzo-p-Dioxin (TCDD) was given to the rats subchronically. It was aim to evaluate the effects on the apoptosis and lipid peroxidation of Retinyl Acetate in the liver and to determine the plasma levels of Vascular Endothelial Growth Factor (VEGF) and Matrix Metalloproteinase-2 (MMP-2) different fixation intervals called as 30, 60, 90 and 120. Apoptosis was determined with assays of Cytochrome c release and DNA fragmentation. Preneoplastic changes in the liver tissues were evaluated histopathologically. In p-DAB+TCDD group there were a decrease apoptosis and an increase lipid peroxidation, VEGF levels and preneoplastic changes beginning from day 60. The increase of MMP-2 levels was shown at day 90 and 120. There was an increase in the apoptosis and also decrease in the lipid peroxidation and levels of VEGF in the Retinyl Acetate treated rats compare to p-DAB+TCDD group at day 60, 90, and 120. Mild preneoplastic changes and decreased MMP-2 were also observed only at day 120. Retinyl acetate may affect the stages of promotion that appeared preneoplastic changes via inducing apoptosis, reducing lipid peroxidation in the liver and decreasing levels of pro-angiogenic molecule VEGF and pro-invasive molecule MMP-2.

Keywords: p-dimethylaminoazobenzene, TCDD, chemoprevention, retinyl acetate, liver


1. Introduction

Retinoids are naturally occurring or synthetic analogues of vitamin A [1]. Retinyl acetate (RA) is the main compound of the vitamin A family. It is one of the esterified forms of retinol founding in foods [2,3]. Retinoids have been reported to exert various effects on cells. They can modulate the growth, differentiation, and apoptosis of normal, premalignant, and malignant cells in vitro and in vivo [4]. Retinoids also have the protective role via antioxidant properties [5,6]. They represent a major class of chemopreventive agents [1]. Retinoids are important in the prevention from various cancers such as skin, mammary gland, liver, lung, prostate, bladder, pancreas and oral cavity of animals which exposed to carcinogenic agents. Retinoids can inhibit promotion through modulating cell differentiation, inhibiting cell proliferation and enhancing apoptosis [4].

Carcinogenesis is a multistage process. These stages are defined experimentally as initiation, promotion, and progression [7]. Initiation (the transformed or initiated cell from normal cell) is an irreversible and rapid stage. Promotion (the preneoplastic cell from initiated cell) is a reversible in its early phases and slower than initiation stage. Progression (the neoplastic cell from the preneoplastic cell) [7,8] is generally irreversible and it is the period between premalignancy and cancer [9].

Chemoprevention, which is defined as natural or synthetic dietary agents, is to reverse, suppress or prevent carcinogenic progression [9]. Chemopreventive agents are known to inhibit one or more stages of carcinogenesis, i.e., initiation, promotion, and progression. It has been reported that the inhibition of cell proliferation and hyperplasia, modulation of cell differentiation and apoptosis, scavenging of reactive oxygen species are among antitumor promoting mechanisms [7].

The liver is the main organ for the metabolism of various toxic chemicals [10]. Hepatic damage and carcinoma is generally associated with exogenous agents. The majority of liver damage or cancer-related diseases are due to continuous exposure or contamination of chemical carcinogens in the food chain [11].

The azo-dye p-DAB is a hazardous compound that used as a coloring agent for polishes and soaps and occasionally as a food additive [12,13]. p-DAB classified as group 2B carcinogen by the International Agency for Research on Cancer with hazardous potential posing a risk to humans and animals [13]. p-DAB is a potential tumor initiator in rodents [14] and has carcinogenic effect in the liver of mice and rats when chronically fed diet mixed
with 0.06% [15]. It has also been reported that hepatocarcinogenesis induced in the several studies which used as initiator p-DAB [13,15,16]. TCDD is a ubiquitous environmental contaminant and toxicant. It is usually released in the environment from several sources such as waste incinerators, herbicides manufacturing, ferrous and non-ferrous metal production, and power generation [17,18]. TCDD accumulates in the food chains due to properties of lipophilic, chemically stable, resistant to biodegradation and persistent in the environment [19,20]. It possesses multiple species- and tissue-specific adverse effects such as carcinogenicity, immunotoxicity, hepatotoxicity, cardiotoxicity, teratogenicity, dermal, endocrine and metabolic alterations [18,21]. TCDD is a potent tumor promoter and its promotion effects have been studied extensively in laboratory animals especially in the liver of female rats [22,23,24]. There are few studies in the liver of male rats. TCDD is a more potent promoter in female Sprague Dawley rats than males [24,25,26]. In Kociba study (1978) using doses of 0, 1, 10, and 100 ng/kg/day for 2 years, TCDD a potent carcinogen in female rats at dose of 100 ng/kg/day but not at lower doses [26]. In another study that treated a liver initiator (N-nitrosodiethyamine), TCDD had a promoting effect at the dose of 0.007 pg/kg/day for 450 days [20]. The observation of promoting effect at this low dose may be associated with the use of a liver initiator substance. Wyde et al. reported that TCDD is capable of promoting the development of preneoplastic foci in male Sprague-Dawley rats when treated with a weekly dose of 700 ng/kg TCDD for 30 weeks after initiation with Diethylnitrosamine. But, induction of GGT-positive altered hepatocyte foci (AHF) was higher in female rats than male rats [25].

In the present study was applied subchronically initiative-p-DAB (0.06%) [13] and promotive-TCDD (100ng/kg/bw) [27]. It was aim to evaluate the effects on the apoptosis and lipid peroxidation of RA on the liver of male rats. TCDD is a more potent tumor promoter and its promotion effects have been studied extensively in laboratory animals especially in the liver of female rats [22,23,24].

2. Material and Methods

2.1. Reagents

Inositol Hexaphosphate and p-dimethylaninoazobenzene were obtained from Sigma Chemical Co., Germany. TCDD was from Accustandard, USA. VEGF, MMP-2 and Cytochrome c (Cyto c) ELISA kits were from R&D Systems, UK. All other chemicals were at the highest purity commercially available.

2.2. Animals

Adult male Sprague Dawley rats, weighing between 275-350 g were used in this study. All animals were acclimatized for 7 days, prior to the commencement of the treatment and allowed free access to food water ad libitum and kept in hygienic condition. Experiments were performed with clearance from the Local Ethical Committee of Eskisehir Osmangazi University, and conducted under overall supervision of the Medical and Surgical Experimental Research Center, Eskisehir Osmangazi University.

2.3. Experimental Design

A group of 24 male rats were used for each of the fixation intervals, namely 30, 60, 90, and 120 days (time after the initiation of TCDD), making a total of 96 animals for the entire study. Each group of 24 rats were divided into four different sets consisting of 6 rats each for control, corn oil control, p-DAB+TCDD, p-DAB+TCDD+RA rats. The first set of rats (control) was allowed normal diet. The second set of rats (corn oil control) was fed normal diet with corn oil (0.25 mL/100 g body weight) by gavage 5 days/week. The third set of rats was kept on a diet mixed with 0.06% p-DAB and was administered TCDD (70 ng/100 g body weight) in corn oil by gavage once a week. The fourth set of rats was fed p-DAB plus TCDD as in the third set of rats, and was fed RA (1 mg/100 g body weight) in corn oil by gavage 5 days/week. Rats were sacrificed at day 30, 60, 90 and 120. TCDD treatment was started one week after initiation of p-DAB. RA treatment was started at the same time with p-DAB. The study was terminated after 120 days of the start of TCDD. After the intracardiac bloods of rats under ether anesthesia were sacrificed by cervical dislocation. The bloods were collected with lithium heparin tubes. Plasma samples were centrifuged at 1,000 xg for 20 minutes and frozen at -80°C for VEGF and MMP-2 assays. Liver tissues were excised, washed three times in ice cold isotonic saline (%0.9) and cut into separate portions for assays and histopathological examination. Histopathological samples were taken in neutral formalin. Other liver portions were stored -80°C until assays.

2.4. Isolation of the Mitochondrial and Cytosolic Fractions

For Cyto c and protein measurements in liver tissue from both the mitochondrial and cytosolic fractions were extracted as follows. Examples were destroyed in 9 volumes of buffer A (in the ice-filled container) with medium-speed Homogenizer. Samples were gently homogenized at medium speed in a Ultra Turrax T25 homogenizer (Janké&Kunkel, IKA-labortechnic Co., Germany) in 9 volumes of buffer A (20 mmol/L HEPES-KOH, pH 7.5, 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 2 mg/mL of aprotinin, 10 mg/ of leupeptin, and 5 mg/mL of pepstatin). The homogenates were centrifuged at 800 xg at 4°C for 10 min, then at 8,000 xg at 4°C for 10 min. The 8,000 xg pellets were washed with buffer A, made soluble in buffer B (10 mmol/L Tris-HCl, pH 8.0, %0.5 Nonidet P-40, and 5 mmol/L CaCl2), centrifuged at 3,000 xg for 10 min, and then were used as the mitochondrial fraction. The supernatant was further centrifuged at 100,000 xg for 60 min at +4°C in an ultracentrifuge (Hanil Ultra 4.0). The resulting supernatant was used as the soluble cytosolic fraction [28].

2.5. Quantification of Cytochrome c in Liver Tissue

The cytosolic and mitochondrial fractions were quantified by ELISA kit (Cytochrome c Quantikine, R&D Systems, UK) following the manufacturer’s instructions. Proteins of the fractions were measured according to the
method of Bradford [29]. Fractions were run in the assay and the resulting nanogram determinations were divided by the protein concentration. The resulting values were expressed as ratio of cytosolic/mitochondrial Cytochrome c.

2.6. DNA Fragmentation Assay

Amounts of fragmented DNA were determined as previously described [30,31] with some modifications. Frozen liver was homogenized in lysis buffer (1:10 w/v; 5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). The homogenate was then centrifuged at 26,000 xg for 25 min at +4°C to separate intact chromatin in the pellet from fragmented DNA in the supernatant. Pellets were resuspended in perchloric acid of 0.5 N and concentrated perchloric acid was added to supernatant samples to a final concentration of 0.5 N. Samples were heated at 90°C for 15 min., and centrifuged to remove protein at 500 xg for 10 min. The resulting supernatants were reacted with diphenylamine for 18 h at room temperature [32] and centrifuged. Absorbance was measured spectrophotometrically at 600 nm. The results were expressed as a percentage ratio of fragmented DNA/intact DNA.

2.7. Lipid Peroxidation Assay

For the lipid peroxidation assay, the liver tissues were minced on glass and homogenized by a glass homogenizer in cold 0.15 N KCl on ice. Then, the homogenate was centrifuged at 2 500 xg (Jouan MR22i) for 10 minutes at +4°C. Supernatant was used for measurement. Lipid peroxidation in liver was estimated by the formation of malondialdehyde (MDA) and measured by the thiobarbituric acid reactive substance (TBARS) method [33]. Measurement of total protein in supernatant was performed according to dye-binding method [29].

2.8. Elisa Assays

The plasma levels of VEGF and MMP-2 were measured by using quantitative sandwich ELISA kits (VEGF Quantikine and MMP-2 Quantikine, respectively, R&D Systems, UK) according to the manufacturer’s protocol.

2.9. Histopathological Examination

Liver specimens were preserved in %10 neutral formalin and dehydrated in a graded alcohol rates. Following xylene treatment, the specimen were then embedded in paraffin blocks and cut into 4 µm thick sections. Sections were stained with hematoxylin and eosin (H & E). All sections are scanned after examined with Olympus BH-2 photomicroscope.

Increased nucleocytoplasmic ratio, compress the surrounding parenchyma, increased number of mitotic figures and multinucleation characterized as the preneoplastic changes [34,35].

2.10. Statistical Analysis

Statistical analysis was performed by using the PASW 18.0 statistical package program. All statistical comparisons were made by means of one-way ANOVA test followed by Tukey LSD post hoc analysis. Data were evaluated using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and was described as mean ± standard deviation (SD). p-values less than 0.05 were considered significant.

3. Results

3.1. Quantification of Cytochrome c in Liver Tissues

Cytochrome c release from mitochondria which is an indicator of apoptosis was quantified by determining of the ratio of cytosolic/mitochondrial Cytochrome c. After 30 days treatment of p-DAB+TCDD was found elevated Cyto c release. But it reduced at next fixation intervals. It was observed an increase in release of Cyto c in RA treated rats when compared to control and p-DAB+TCDD group at day 60, 90 and 120 Figure 1A.

![Figure 1. (A-B): Histograms showing of Cytochrome c release (cytosol/mitochondria) (1A), and DNA fragmentation (supernatant/pellet x100) (1B) in different series at different fixation intervals. Versus group control: *; p < 0.05 **; p < 0.01 ***; p < 0.001, versus group p-DAB + TCDD: ##; p < 0.01 ###; p < 0.001](image-url)
### 3.2. DNA Fragmentation

Another indicator of apoptosis, DNA fragmentation similar to Cyto c release elevated in p- DAB+ TCDD group at day 30 and then reduced at day 60, 90 and 120. After treatment RA increased DNA fragmentation compare to p- DAB+ TCDD group at day 60, 90 and 120 Figure 1B.

### 3.3. Lipid Peroxidation (LPO) Assay

Table 1 show that levels of LPO (nmol MDA /mg protein) in the liver of control and experimental animals. Increased lipid peroxidation in p-DAB+TCDD treated rats after treatment RA increased DNA fragmentation compare to p- DAB+TCDD group at day 60, 90 and 120.

### 3.4. Elisa Assays

Figure 2A and Figure 2B shows plasma levels of VEGF and MMP-2 in the control and experimental animals. In the p-DAB+TCDD treated group increased the plasma levels of VEGF and MMP-2 at the second and third fixation interval, respectively. The increase of MMP-2 was later than the increase of VEGF. RA treatment reduced VEGF levels when compared to p-DAB+TCDD group at day 60, 90 and 120, and MMP-2 levels at day 120.

### 3.5. Histopathological Examination

Table 2 shows histopathological changes in different groups. No preneoplastic changes were observed in p-DAB+TCDD group at day 30. But starting from the second fixation interval appeared the preneoplastic changes. Histopathological examination showed the preneoplastic changes that characterized increased nucleocytoplasmic ratio, compress the surrounding parenchyma, increase number of mitotic figures and multinucleation. It was also observed eosinophilic nucleolus in hepatocytes and hidropic degeneration in the liver tissues of p-DAB+TCDD group. In the RA treated rats were close to normal architecture the liver tissues all fixation intervals except mild preneoplastic changes at day 120. Representative photomicrographs of histopathological changes in groups are shown in Figure 3.

In the present study we aim to evaluate the anti-promotive effects of RA in the subchronically carcinogen treated rats at different fixation intervals. It was also examined the tissues of livers histopathologically.
Figure 3. (a-f): Histopathological studies in the liver of corn oil control and experimental animals. Liver of corn oil control animals revealed a normal architecture (a). In the RA treated rats were observed mild preneoplastic changes at day 120 (b). It was shown slightly degeneration and enlarged sinusoidal spaces in p-DAB+TCDD treated rats at day 30 (c). In p-DAB+TCDD treated rats were also observed preneoplastic changes at day 60, 90 and 120 (d-f). Liver tissues were stained with Hematoxylin and Eosin; original magnification (X10).

<table>
<thead>
<tr>
<th>Preneoplastic lesions</th>
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<td>Corn oil</td>
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<td>p-DAB+TCDD</td>
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<td>p-DAB+TCDD+RA</td>
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+ = mild; ++ = moderate; +++ = severe; - = no change, n=6

4. Discussion

Retinoids are natural or synthetic compounds. They have potential chemopreventive agents that could inhibit promotion through properties of growth inhibitive, differentiative, antioxidative and apoptotic [1,6,36]. When added vitamin A (retinyl acetate, retinoic acid and retinol) during the initiation and promotion phase is exhibited inhibitory activity [36,37]. If retinoids are given in the early stages of carcinogenesis, they cause regression of premalignant lesions of the skin [36]. The inhibition of hepatocarcinogenesis has been observed in the retinyl acetate and retinoic acid fed rats. Previous studies have indicated that vitamin A deficiency in experimental animals has been associated with a higher incidence of cancer and with increased susceptibility to chemical carcinogens [4]. There are many studies related to vitamin A homeostasis modified by TCDD. TCDD affect cellular utilization and deposition in the liver of vitamin A. It was shown that reduced retinyl esters and reduced or increased levels of retinoic acid in the liver depending on the dose and the exposure time of TCDD in female and male rats [38,39]. We have not determined the levels of liver retinoids due to the aim of this study not to evaluate the effects of TCDD on the levels of retinoids. But according to the results of our study we can say that the dose of RA that used in this study had the protective ability against the effects of p-DAB+TCDD.

One of the protective capabilities of retinoids is via antioxidant properties and they protect against lipid peroxidation [5,6,40]. In this study, it was shown lipid peroxidation by measuring the levels of MDA that a marker of lipid peroxidation in the liver tissues. The levels of MDA reduced in RA treated group compared to p-DAB+TCDD group at all fixation intervals. In p-DAB+TCDD group increased the levels of MDA versus control group at day 60 and remained high in the next fixation intervals. Antioxidant mechanisms as well as the modulation of apoptosis are among tumor promoting mechanism [7]. Apoptosis is a form of cell death which inducible by both endogenous and exogenous stimulus. Apoptosis can occur in response to exposure to any kind of hepatocarcinogen [41]. The inhibition of apoptosis has an important role in tumor promotion. After treatment with genotoxic liver carcinogen (as initiator), subsequent administration of certain non-genotoxic agents (promoter) may lead to the clonal expansion of putative preneoplastic.
cells. The expansion of those clones is correlated with an increased occurrence of benign and malignant liver tumors. Inhibition of apoptosis has been demonstrated in studies with liver tumor promoters, such as Phenobarbital and TCDD [42,43]. In the present study, assays of Cytochrome c release and DNA fragmentation in the liver tissues were used as markers of apoptosis. It was shown that an increase Cyto c release and DNA fragmentation in p-DAB+ TCDD group compare to control at day 30. Apoptosis inhibited at the next fixation intervals. Initially induced then inhibited apoptosis may be related with disruption the balance between apoptosis and proliferation of cells [41] and effect of promotive-TCDD. Retinoids have shown their effects through biologically active retinoid derivatives, retinal and retinoic acid [39]. It was known retinoic acid has a direct effect on mitochondrial membrane; it may decrease the membrane potential and cause Cyto c release and organelle swelling. Retinoic acid induces the apoptosis through Cyto c release and DNA fragmentation [44,45]. In our study, the release of Cyto c increased in RA treated group at all fixation intervals. RA treatment ameliorated DNA fragmentation compared to p-DAB+TCDD group at day 60 and 90, and increased at day 120. However DNA fragmentation was lower in RA treated group compare to the control group at day 60 and 90. Low DNA fragmentation in RA treated group may be observed in relation to the size of the fragments retained in the supernatant. It was known DNA fragmentation into oligonucleosomal ladders is characteristic of apoptosis, but not all cells undergo such extensive DNA fragmentation. Actually, fragmentation of DNA into kilobase-size fragments appears to be an early event in apoptosis and later occurs complete digestion of DNA into multiples of nucleosomal size fragments [46].

Angiogenesis is essential for both tumor growth and metastasis. There is highly correlation between angiogenesis and the expression of VEGF in carcinoma [47]. VEGF is the most potent and specific growth factor for both angiogenesis and vasculogenesis which is induced by inflammatory stimuli and hypoxia [48,49]. VEGF is a primary inducer of angiogenesis [49]. It stimulates proliferation, migration, survival, and secretion of matrix-degrading enzymes [50]. The increased VEGF may contribute to the growing process in hepatocarcinogenesis through angiogenesis [51]. Tumor invasion and metastasis require the destruction of the extra cellular matrix (ECM) and basement membrane to allow cell migration by matrix metalloproteinases [27,52]. MMPs are a family of Zn2+ and Ca2+ dependent endopeptidases. They affect various cellular processes such as proliferation, differentiation, migration and adhesion [16]. There are several studies showing that increased or decreased VEGF synthesis by TCDD. The nature of VEGF modulation by TCDD is not very clear. The responses of VEGF to TCDD exposure depends on the species and cell type [53]. In the studies about the effects on MMP-2 expression of TCDD has shown that TCDD induced the expression of MMP-2 through AhR pathway. TCDD may influence tumor promotion through altering the expression of the matrix metalloproteinases [27]. Subchronic p-DAB administration has also induced the expressions of VEGF and MMP-2 in hepatoma [54]. In the present study, the increase in response to carcinogen administration of VEGF that is an angiogenic molecule [48] observed before the increase of MMP-2. In p-DAB+TCDD group increased plasma levels of VEGF second fixation interval and next ones. The increased levels of MMP-2 were observed the fixation interval which followed the increase in VEGF. These results maybe suggest that VEGF may have a role in the increase of MMP-2. In the fixation intervals that increased VEGF were shown suppression of apoptosis too. Several studies showed that in vivo apoptosis is correlates with a downregulation of tumor VEGF expression and VEGF protects endothelial cells from apoptosis, by inducing the antiapoptotic gene, Bcl-2 [55,56]. In the fixation intervals that VEGF has increased and apoptosis has decreased, there was also increase in the levels of MDA and preneoplastic changes.

The inhibition of proangiogenic and proinvasive molecules is also discussed in the concept of chemoprevention [47,54]. The inhibition of tumor-induced angiogenesis is one of the anti-tumor activities of retinoids. Retinoic acid inhibits VEGF-induced angiogenesis [57]. Retinoids have also been shown to inhibit metastasis in a variety of model systems. Retinoic acid has reduced tumor cell invasion by decreasing mRNA, protein levels or enzyme activity of MMP and retinol has decreased the activity of MMP-2 and MMP-9 [52]. We were observed that a decrease in the plasma levels of VEGF in RA treated group at all fixation intervals except day 30 and levels of MMP-2 at day 120. In the fixation intervals that VEGF decreased and apoptosis increased were determined a decrease in the levels of MDA in the RA treated group. RA treatment caused to the only mild preneoplastic changes in the liver at day 120.

In conclusion, it was determined that caused histopathologically preneoplastic changes in the liver of male SD rats of p-DAB+TCDD at the beginning from second fixation interval. In the fixation intervals that observed preneoplastic changes there was an increase lipid peroxidation, plasma levels of VEGF and MMP-2 and a decrease apoptosis. In the RA group that treated at the same time with p-DAB were close to normal architecture the liver tissues all fixation intervals except mild preneoplastic changes at day 120. The data related with increased apoptosis and decreased lipid peroxidation, plasma levels of VEGF and MMP-2 indicated that RA can have the effect of anti-promotive. Pharmacological doses of RA may be important in chemoprevention in the case of exposure to carcinogens such as TCDD which can decrease retinoid content of the liver [38,39]. Further studies that contain the different pathways and mechanisms related to the promotion will appear to the role related with anti-promotive effect of RA.

Acknowledgement

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Conflict of Interest

We have no conflict of interest.
Abbreviations

Cytochrome c (Cyto c), Lipid Peroxidation (LPO), Malondialdehyde (MDA), Matrix Metalloproteinase-2 (MMP-2), p-dimethylnitrosamine (p-DAB), Retinol acetate (RA), 2, 3, 7, 8-Tetrachlorodibenzo-p-Dioxin (TCDD), Vascular Endothelial Growth Factor (VEGF).

References


