Retinoic acid repressed the expression of c-fos and c-jun and induced apoptosis in regenerating rat liver after partial hepatectomy

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Abstract

Retinoic acid (RA), which was injected within 4 h after partial hepatectomy (PH), inhibited DNA synthesis in regenerating liver. The inhibition was accompanied by apoptosis, evidenced by in situ end labeling and gel electrophoresis of DNA fragmentation. Characteristic DNA fragmentation was obvious at 4 h and reached a maximum at 8 h after injection. Northern blot analysis revealed that RA repressed the expression of c-fos and c-jun at 15 and 30 min with the up-regulation of retinoic acid receptor γ (RARγ) and RARβ at 2 h after PH. The transglutaminase II mRNA level and activity were increased by RA injection at 4 h and 8 h after PH, respectively. The mRNA levels of thymidylate synthase and thymidine kinase, which are rate determining enzymes of DNA synthesis, decreased in RA injected rats. No change was seen in the expression of p53 and p21WAF1/CIP1 which have been suggested to participate in the apoptosis process. These results suggest that RA exerts the antiproliferative activity only on the early stage of liver regeneration accompanied by the repression of c-fos and c-jun expression and induction of apoptosis. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Retinoic acid; Apoptosis; c-fos; c-jun; Liver regeneration; Rat

1. Introduction

Retinoic acid (RA), a physiological metabolite of vitamin A, has potent effects on many biological functions, such as proliferation, differentiation and fetal development [1,2]. RA has been used as chemotherapeutic agents because of its strong antiproliferative activity against certain types of cancers [3].

Similarly, many tissue culture cells undergo differentiation in response to RA treatment. RA stimulates apoptosis in cultured HL60 human promyelocytes, neuroblastoma and breast cancer cell lines [4-6]. Thus, the antiproliferative effects of RA has been studied primarily in tumor cell lines and significant differences exist between cell types. Very little is known about the effects of RA on normal cells. The purpose of this study was to examine the effects of RA on the normal cell proliferation.

The regenerating liver following two-thirds partial hepatectomy (PH) in the rat is considered one of the most feasible models to study the cellular changes occurring in vivo in different phases of the cell cycle of normal cell proliferation because of the relevant synchronism of the first cell cycle, the timing of
which has been well established [7]. After PH, most remaining hepatocytes promptly enter and progress in the cell cycle in a synchronous manner [8]. The first ‘priming’ stage, in which hepatocytes undergo a transition from a resting state (G0) to one in which they become capable of proliferating if the appropriate inducer is present (G1), is characterized by an increase in the expression of the immediate early response genes such as c-fos and c-jun [9,10]. The priming stage encompasses the first 4–6 h after PH. The ‘primed’ hepatocytes then enter a ‘progression’ stage characterized by an increase in p53 mRNA. After a prereplicative phase of the priming and progression stage lasting 12–16 h, the expression of thymidylate synthase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21) which are rate determining enzymes of DNA synthesis [11], is induced at the G1/S boundary [12,13]. DNA synthesis starts at about 18 h after PH and peaks at 24 h. Using this model, we investigated the effects of RA on the normal cell proliferation and the expression of cell cycle associated genes.

The induction of apoptosis by retinoids is related to cell growth and differentiation in various ways, depending on cell type. Growth arrest by retinoids can lead to either terminal differentiation or apoptosis [14]. Retinoids first induced differentiation, and then the differentiated cells underwent apoptosis as exemplified by HL-60 myeloid leukemia cells [4], while apoptosis was induced in a process that was independent of differentiation in neuroblastoma cells [5]. In F9 embryonal carcinoma cells and p39 myelomonocytic leukemia cells, retinoids induced differentiation and apoptosis concurrently [15,16]. A synthetic retinoid induced G0/G1 arrest and apoptosis in human breast cancer cells [17]. G1 arrest and apoptosis by RA were also observed in human neuroblastoma cells [18]. RA caused accumulation of Hep 3B hepatocellular carcinoma cells in sub-G1 phase and induced apoptosis [19]. These suggest that the starting point for apoptosis could be related to specific phase of the cell cycle as well as the differentiation stage. It is important to elucidate when and how RA switches on the apoptosis and to define this process in terms of changes in expression of specific genes.

In the present study we evaluated the antiproliferative effect of RA and the RA induced apoptosis focusing on the cell cycle related gene expression during liver regeneration. The results showed that RA exerted anti-proliferative activity only at the priming stage of liver regeneration accompanied by the repression of the c-fos and c-jun expression and induction of apoptosis.

2. Materials and methods

2.1. Materials

The reagents were purchased from the following sources: [methyl-3H]thymidine (60 Ci/mmol), ICN Biomedicals; [5-3H]deoxyuridine monophosphate (14.8 Ci/mmol), Amersham; [2,3-3H(N)]putrescine (80 Ci/mmol), ARC; DIG RNA labeling kit and DIG luminescent detection kit, Boehringer Mannheim; Oligo(dT)cellulose (type 3), Collaborative Research; Gene screen nylon membrane, NEN Research Products. All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats weighing 180–200 g were used for all experiments. The animals were kept in temperature controlled rooms with 12 h alternating light and dark cycles and given commercial laboratory chow (MF, Oriental Yeast, Osaka, Japan) and water ad libitum. Two-thirds partial hepatectomy (PH) was performed by the procedure of Higgins and Anderson [20]. RA (15, 2 or 0.5 mg/kg body weight), dissolved in corn oil/dimethylsulfoxide (1:1), was intraperitoneally injected immediately or at indicated times after PH. Control rats received the same quantity of the vehicle as the experimental animals. The rats were killed under diethyl ether anaesthesia and their livers were excised at indicated times.

2.3. Determination of the enzyme activities and contents of DNA, RNA and protein

The excised liver was homogenized with 5 volumes of 50 mM Tris-HCl buffer (pH 7.3) containing 0.25 M sucrose, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. TS and TK activities of the supernatant fraction of a
20% liver homogenate were determined and expressed as pmol of product formed/min per mg protein at 37°C as described previously [11]. The activity of serum glutamate-oxaloacetate transaminase (GOT; EC 2.6.1.1) was measured spectrophotometrically utilizing diagnostic kits (Wako, Osaka, Japan) and expressed as IU at 25°C. The TGase II activity of the liver homogenate was measured by detecting the incorporation of [3H]putrescine into N,N'-dime-thylcasein [21]. Protein was measured by the method of Lowry et al. [22] with bovine serum albumin as standard. The DNA and RNA contents of the liver were measured by the diphenylamine [23] and orcin reactions [24], respectively, after extraction with trichloroacetic acid according to the procedure of Schneider [24]. Statistical analyses of data were done with one-way analysis of variance with pairwise comparison by the Bonferroni method [25].

2.4. In situ end labeling of 3'-OH ends of DNA fragments

Paraformaldehyde fixed paraffin embedded liver sections, obtained from the regenerating liver of control and RA injected rats at 8 h after PH were processed for in situ detection of DNA fragmentation by the terminal deoxynucleotidyl transferase (TdT) mediated nick end labeling technique (TUNEL) [26] using the In Situ Cell Death Detection kit (Boehringer-Mannheim, Germany). Briefly, deparaffinized tissue sections were enzymatically labeled with fluorescein-nucleotide via terminal deoxynucleotidyl transferase and subsequently exposed to horseradish peroxidase conjugated anti-fluorescein antibody. Staining was developed in diaminobenzidine and sections were counterstained with Mayer’s hematoxylin.

2.5. Isolation and gel electrophoresis of DNA

Liver was homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 0.5% sodium dodecyl sulfate, and incubated overnight with proteinase K (200 µg/ml) at 50°C. After RNase digestion, DNA was extracted with NaI solution [27] and electrophoresed on 2% agarose gel using a buffer containing 40 mM borate (pH 7.4) and 1 mM EDTA.

2.6. Isolation and Northern blot analysis of RNA

Total RNA was extracted from the liver in 4 M guanidinium isothiocyanate [28] and fractionated by affinity chromatography on an oligo(dT)-cellulose column to obtain poly(A)^+ -rich RNA. The concentrations of RNA samples were measured by absorbance at 260 nm. The purities of RNA samples were determined by the ratio of A260nm/A280nm (> 1.8) and by electrophoresis in formaldehyde-agarose gels stained with ethidium bromide. The RNA preparations were denatured and electrophoresed on 1.2% agarose/2.2 M formaldehyde gels. After separation, the RNA was transferred to gene screen membranes by capillary blotting. Hybridization was carried out using Dig labeled RNA probes and the chemiluminescent signals were quantitated by a densitometer as described previously [11]. Equal lane loading and the transfer efficiency of RNA samples were verified by the intensity of ethidium bromide fluorescence of the rRNA on the gel and the filter. The comparable levels of albumin mRNA which do not vary during 24 h after PH were also confirmed in RNA samples used for blotting [29,30].

2.7. Preparation of RNA probe

The EcoRI/NotI fragment (1.8 kb) of the rat TGase II cDNA, the EcoRI/PstI fragment (1.8 kb) of c-jun (ATCC 63026), the PstI fragment (1.0 kb) of c-fos (ATCC 41040), the EcoRI/EcoRV fragment (0.86 kb) of mouse RARα cDNA, the BamHI fragment (1.8 kb) of mouse RARβ cDNA, the BamHI/EcoRI fragment (1.8 kb) of mouse RARγ cDNA, the BamHI fragment (0.9 kb) of mouse RXRα cDNA, the EcoRI/HindIII fragment (1.4 kb) of mouse RXRβ cDNA, the EcoRI fragment (1.5 kb) of mouse RXRγ cDNA, the EcoRI fragment (0.85 kb) of mouse p21 cDNA, the HindIII/AccI fragment (1.3 kb) of mouse p53 cDNA (RDB 1284), the PstI fragment (0.7 kb) of mouse TS cDNA, the EcoRI/PstI fragment (0.8 kb) of rat TK cDNA or the PstI fragment (0.5 kb) of rat albumin cDNA were subcloned into plasmid Bluescript. After linearization of the plasmid, T7 RNA polymerase was employed to obtain run-off transcripts of the antisense strands. Transcription and labeling were performed utilizing
the commercial DIG RNA labeling system according to the manufacturer’s instructions.

3. Results

3.1. Effects of RA on the activities of TS and TK, the DNA, RNA and protein content, and the liver weight of regenerating liver

The activities of TS and TK at 24 h after PH are shown in Table 1. The TS activity increased to about 8-fold the normal value (resting in G0 state; just after PH). When RA was injected immediately after PH at a dose level of 15 mg/kg body weight, TS activity decreased to 33% of the control value. TK activity was also depressed by the injection of RA to 17% of the control. The decreases in TS and TK activities were followed by significant reduction of the increase in liver DNA content. The RNA content and gross parameters such as protein content and liver weight were also reduced significantly. These inhibitory effects on TS and TK activity and DNA contents were also observed by the administration of RA at 2 h or 4 h after PH. When RA was injected at 8 h after PH, however, the regenerative inhibition was not observed at all. These results indicated that RA exerted the antiproliferative activity on regenerating liver at the priming stage within 4 h after PH. When the dose level of RA was reduced to 2 mg/kg body weight, the inhibitions of TS and TK activities and DNA content were less but significant. At the dose level of 0.5 mg of RA/kg body weight, the inhibitory effects on liver regeneration were not observed. These results showed that the inhibition of liver regeneration by RA was dose dependent as well as time dependent.

![Fig. 1. Analysis of DNA fragmentation by agarose gel electrophoresis.](image)

RA (15, 2 or 0.5 mg/kg body weight) was intraperitoneally injected immediately after PH. Genomic DNA was isolated from liver of the control (lanes 1–5) and RA injected (lanes 6–10) rat at 2, 4, 8, 12, and 24 h after PH. Lane M contained HindIII digested \( \lambda \) DNA as molecular size marker. Lanes 1–5 were DNA from the control at 2, 4, 8, 12 and 24 h after PH, respectively. Lanes 6–10 were DNA from the RA injected rat liver at 2, 4, 8, 12, and 24 h after PH, respectively. The result presented here is typical of four separate experiments.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Injection time (h after PH)</th>
<th>Enzymatic activity (pmol/min/mg of protein)</th>
<th>Total liver content (mg)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TS</td>
<td>TK</td>
<td>DNA</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>82.4 ± 5.0</td>
<td>373.1 ± 34.6</td>
<td>7.69 ± 0.15</td>
</tr>
<tr>
<td>RA</td>
<td>15</td>
<td>0</td>
<td>27.1 ± 5.1*</td>
<td>63.6 ± 13.4*</td>
<td>5.93 ± 0.37*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>43.2 ± 8.6*</td>
<td>86.7 ± 12.0*</td>
<td>6.16 ± 0.36*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>54.6 ± 3.4*</td>
<td>157.1 ± 12.1*</td>
<td>6.32 ± 0.25*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8</td>
<td>73.4 ± 2.2</td>
<td>306.3 ± 36.3</td>
<td>7.48 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>62.7 ± 4.0*</td>
<td>238.1 ± 23.8*</td>
<td>7.08 ± 0.15*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0</td>
<td>75.7 ± 5.1</td>
<td>361.0 ± 72.3</td>
<td>7.41 ± 0.45</td>
</tr>
<tr>
<td>Normal (just after PH)</td>
<td></td>
<td></td>
<td>10.4 ± 0.8*</td>
<td>34.4 ± 6.9*</td>
<td>5.81 ± 0.20*</td>
</tr>
</tbody>
</table>

RA (15, 2 or 0.5 mg/kg body weight) was intraperitoneally injected at 0, 2, 4 or 8 h after PH. At 24 h after PH, the activities of TS and TK, the content of DNA, RNA, and protein, and liver weight were determined as described in Section 2. Values are means ± S.E.M. of 5–8 rats.

*Significant difference from the control (P < 0.05).
3.2. Apoptosis induced by RA

RA, injected immediately after PH, induced the DNA fragmentation as shown in Fig. 1. Agarose gel electrophoresis revealed a characteristic ladder pattern of DNA in the RA injected rat liver (Fig. 1). DNA isolated from the control rat liver yielded bands only in the high molecular weight region. A

Fig. 2. In situ end labeling of the apoptotic bodies in a liver section of RA injected (upper) and control rat (lower) at 8 h after PH. RA (15 mg/kg body weight) was intraperitoneally injected immediately after PH. Paraformaldehyde fixed paraffin embedded liver sections, obtained from the regenerating liver of RA injected and control rats at 8 h after PH were processed for in situ detection of DNA fragmentation by TUNEL as described in Section 2. TUNEL stained nuclei are marked by arrows. Hematoxylin counterstaining. The results presented here are typical of four separate experiments. Magnification ×400.
time course study of DNA fragmentation showed that significant DNA cleavage started at 4 h and peaked at 8 h after PH in RA injected rat liver. Fig. 2 is a representative example of in situ labeling of apoptotic cells in a liver section from the control and RA injected rat at 8 h after PH. TUNEL positive staining was observed in nuclei and nuclear fragments with the morphological characteristics of apoptosis in the RA injected rat liver, with negligible background staining in the control. The identification of stained apoptotic bodies was confirmed by specific morphological criteria including nuclear condensation, cytoplasmic compaction and detachment from neighboring cells [31]. In the present evaluation, hepatocytes with necrotic morphology were a rare occurrence and foci of inflammatory cells were absent under light microscopy after hematoxylin and eosin staining.

3.3. Effects of RA on the activities of serum GOT and liver TGase II

As a functional assay substantiating that the effect of RA is apoptotic and not necrotic, GOT release was determined as a marker of membrane integrity. The administration of RA had no significant effect on serum GOT activity during liver regeneration as shown in Table 2, indicating that necrosis was not caused by RA up to 24 h after PH at the dose level employed.

A rise in tissue transglutaminase activity has been described as a prominent feature of some cells undergoing chemically induced apoptotic cell death [32–34]. To examine the relation between apoptosis and transglutaminase activation in our system, the time course of TGase II activity of the liver was measured during regeneration after PH. The TGase II activity significantly increased over control values after 8 h, at which the DNA fragmentation peaked (Fig. 1), and kept on increasing to 24 h as shown in Table 2. These results showed that RA induced apoptosis with concomitant increase of TGase II activity during liver regeneration.

3.4. Effects of RA on gene expression during liver regeneration

To address that the increase in TGase II activity occurred at the transcriptional level, the mRNA levels of TGase II in RA injected rat were compared with those of the control during liver regeneration. Northern blot analysis showed that the RA injection caused marked increases in TGase II mRNA levels at 4 h and the increasing continued up to 24 h to about 15–20-fold the normal value (Fig. 3A; lanes 7–10). This indicated that the up-regulation of TGase II activity by RA injection was caused by the increase in its mRNA level.

The effects of RA on p53 and p21 mRNA levels were examined. The level of p53 mRNA increased at 8 h peaking at 8–12 h after PH (about 3–5-fold over the normal value) as shown in Fig. 3A. RA injection did not cause a significant change in the p53 expression pattern during liver regeneration (Fig. 3A, lanes 6–10). p21 mRNA was barely detectable in normal liver. After PH, a biphasic pattern of p21 expression
was observed as shown in Fig. 3A. The p21 mRNA levels increased at 2–4 h, declined to the normal level at 8–12 h, and increased again at 24 h after PH. The level and pattern of p21 mRNA in RA injected rats were similar to those in the control (Fig. 3A, lanes 6–10).

The changes of the mRNA levels of RARα, β, γ and RXRα, β, γ by RA injection were investigated (Fig. 3A). The RARα probe hybridized to two mRNA species of 3.7 kb and 2.8 kb as shown in Fig. 3A. The RARα mRNA levels decreased at 2 h and returned to the normal level at 12 and 24 h after PH in the control, while the constant level was observed in RA injected rats. The RARβ mRNA (3.3 kb and 3.0 kb) levels decreased at 8–12 h and recovered to the normal level at 24 h after PH. In contrast to the control, the RARβ mRNA level in RA injected rat markedly increased at 2 h after PH to about 6-fold the corresponding control value. The RARγ mRNA (3.3 kb) was barely detectable in the normal liver and increased to about 10-fold the normal at 8 h to 24 h after PH. In RA injected rats, the RARγ mRNA level remarkably increased at 2 h after PH. Densitometric analysis showed that RA increased the RARγ mRNA level at 2 h to 6–8-fold the corresponding control value. The levels and patterns of RXRα, β, γ mRNA expression in RA injected rats were similar to those in the control as shown in Fig. 3A.

The effects of RA on the expression of the immediate early response genes c-fos and c-jun were studied. The c-fos mRNA levels were barely detectable in the normal liver and increased rapidly and markedly at 15 min and 30 min after PH as shown in Fig. 3B (lanes N, 1 and 2). RA remarkably suppressed these increases (Fig. 3B, lanes 5 and 6). Scanning densitometry showed that the level of c-fos mRNA in RA injected liver decreased to about 20% of the control. The levels of c-jun transcripts increased to about 10-fold the normal value at 15 min and 30 min after PH as shown in Fig. 3B (lanes N, 1 and 2). RA also blocked the induction of the c-jun mRNA to 20–30% of the control. These results clearly demonstrated that RA repressed the expression of c-fos and c-jun in regenerating liver at the priming stage.

The effects of RA on the expressions of RARα, β and γ at the early stage within 2 h during liver regeneration were also examined (Fig. 3B). The levels of RARα mRNA decreased at 1 and 2 h after PH in the control but did not significantly changed in RA injected rats during 2 h after PH. The RARβ mRNA levels of RA injected rat were similar to those of the control until 1 h and markedly increased (8–12-fold the normal) at 2 h after PH. The RARγ mRNA levels also markedly increased from 1 h to 2 h (6–8-fold the normal) by RA injection.

The mRNA levels of TS and TK were barely detectable in the normal quiescent liver and increased at 24 h after PH as previously reported [11–13]. Northern blot analysis showed that RA inhibited the increase in 1.3 kb TS mRNA level at 24 h to 30% of the control value proportionally to the TS activity (Fig. 3C). The levels of 2.6 kb and 1.1 kb mRNA of TK were also reduced by RA injection in proportion to the decrease in the activity.

4. Discussion

This study clearly demonstrated that RA exerted the antiproliferative activity only on the early stage
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A

TGase II

p53

p21

RARα

RARβ

RARγ

RXRα

RXRβ

RXRγ

albumin

N 1 2 3 4 5 6 7 8 9 10
Fig. 3 (continued)
of regenerating liver and induced apoptosis. When RA was injected within 4 h after PH, it decreased the activities of the rate determining enzymes of DNA synthesis, TS and TK and the subsequent DNA content at 24 h after PH (Table 1). The injection of RA at 8 h after PH did not cause the inhibitory effects on liver regeneration at all. These indicated that RA exerted its antiproliferative activity on the liver cells only at the ‘priming’ stage (G0/G1 transition) during regeneration. The regenerative inhibition was accompanied by apoptosis. The characteristic DNA fragmentation was observed as early as 4 h and peaked at 8 h after PH (Fig. 1). The results of in situ end labeling also showed appearance of apoptotic cells with specific morphological criteria (Fig. 2). The possibility of necrosis was excluded by the morphological analysis and serum GOT level. These results showed that RA induced apoptosis in liver during normal cell proliferation as in many tumor derived cultured cell systems [4–6,15–18], although the dose used in this study was at the pharmacological level.

RA induced apoptosis was preceded by the inhibition of the decrease in the RARα expression and by the upregulation of RARγ and RARβ mRNA levels within 2 h after PH. The decrease in the RARα mRNA levels at 1 and 2 h after PH was not observed in RA injected rats (Fig. 3B). The changes in RARα, β, γ expression after PH suggest the physiological role of RA in liver regeneration. The decrease in RARα expression at the early stage of liver regeneration may be needed to induce the proliferation. In liver cell proliferation, it is also suggested that the RARα is involved in the control of proliferation [35,36], whereas RARβ controls events that lead to cellular differentiation [35]. The physiological roles for RA induced increases in RARβ and γ expression are not known. RARβ contains the retinoic acid responsive element and is inducible, while RARα is not inducible [37,38]. The administration of RA to normal rats caused the overexpression of RARβ transcripts, although the levels of RARα and RARγ mRNAs were not affected [37,39]. However, in this study, the increase in the expression of RARγ as well as RARβ by RA injection was observed within 2 h after PH as shown in Fig. 3B. RARγ mRNA was barely detectable in normal liver (Fig. 3 and [39]). The tissue distribution of the RARγ transcripts is restricted [40]. The phenotypic changes demonstrated by null mutant mice of all RAR isoforms may be explained by the perturbation of apoptosis during development [41,42]. The experiments using receptor selective retinoids showed that the induction of apoptosis in neuroblastoma cells was under the control of RARα and RARγ [43]. Retinoids induced apoptosis in T cells through the stimulation of RARγ and the RARγ mediated apoptosis was accompanied with the increase in the expression of TGase II [44]. The TGase II mRNA level and activity of RA injected rat liver also increased at 4 h and 8 h after PH, respectively (Table 2 and Fig. 3A). Taking these results together, the increase in RARγ expression observed suggests that RARγ may be involved in RA induced apoptosis in regenerating liver.

The increase in TGase II expression by RA was associated with apoptosis during liver regeneration. RA injection also increased TGase II activity, but did not cause apoptosis in normal liver [45,46]. These results suggest that RA induced apoptosis is triggered in actively cycling cells, not in nonproliferating and fully differentiated cells. This may be important in explaining some of the antitumor effects of RA.

In agreement with the result that RA act on the liver cell at early stage during regeneration, the expressions of the immediate early response proto-oncogenes, c-fos and c-jun were markedly repressed by RA (Fig. 3B). The down-regulation of c-fos expression by RA associated with growth arrest was also reported in a fibrosarcoma cell line [47]. In addition to the repression of the c-fos mRNA level, the reduction of c-jun expression observed suggests decreases in the products of these genes, Fos and Jun proteins, the principal AP-1 components. In fact, it is reported that AP-1 activity increased proportionally to the increase in c-jun mRNA levels in regenerating liver [48]. The AP-1 transcription factor has been shown to be essential for DNA replication [49]. In this study, the levels of TS and TK mRNA which are induced at G1/S were also decreased by RA in accord with the down regulation of c-fos and c-jun mRNA (Fig. 3C). Furthermore, a correlation between the presence of AP-1 DNA binding activity and the repression of the cell death pathway was reported in lymphocytes [50]. On the other hand, RA caused accumulation of Hep 3B cells in sub-G1 phase during apoptosis and the G0/G1 transition was
suggested to be the main control check point [19].
These suggest that the expression of c-fos and c-jun and the subsequent AP-1 activity play an essential role in determining whether a cell will progress through the cell cycle or undergo apoptosis. It is possible that the repression of c-fos and c-jun expression by RA induces apoptosis during liver regeneration after PH.

Molecular details of apoptosis pathway are still poorly defined, but recent studies indicate that apoptosis is regulated by the balance of various molecules including tumor suppressor genes, proto-oncogenes, and cell cycle regulators. The p53 tumor suppressor gene and the cyclin dependent kinase inhibitor p21\(^{WAF1/CIP1}\) are known to participate in apoptosis [51]. In this experiment, however, RA did not influence the transcription of p53 and p21\(^{WAF1/CIP1}\) gene. There was a striking similarity with respect to the gene expression of these negative regulators of the cell cycle between proliferative processes and RA induced apoptosis processes in regenerating liver.

In conclusion, our paper has provided evidence that RA is able to induce apoptosis accompanied by the repression of the expression of c-fos and c-jun during liver regeneration after PH and the induction of apoptosis may contribute to the antiproliferative activity of RA against regenerating liver at the early stage. Further study remains necessary, however, to clarify the molecular mechanisms regulating RA induced suppression of the proto-oncogene expression and apoptosis.

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