[総説] Effects of acyclic retinoid on cell cycle progression and signal transduction

Effects of acyclic retinoid on cell cycle progression and signal transduction

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ABSTRACT

Acyclic retinoid (ACR), a novel synthetic retinoid, can prevent the recurrence of hepatomas after surgical resection of primary tumors, but the molecular mechanisms by which ACR exerts anti-tumor effects are not known. In recent studies we found that growth inhibition of human hepatoma cells by ACR is associated with induction of $p21^{CIP1}$ and inhibition of expression of cyclin D1. β-Catenin stimulates cyclin D1 promoter activity through T cell factor (TCF) in the HepG2 human hepatoma cell line, and this activity is inhibited by ACR. We also found that ACR activates retinoic acid receptor β and induces transcriptional activation of $p21^{CIP1}$ in human hepatoma and squamous cell carcinoma cells. These novel effects of ACR suggest that this agent might be useful in the chemoprevention and therapy of various malignancies. These studies and their implications are summarized and discussed in this article. *Ryukyu Med. J., 23(1,2) 1～9, 2004*

Key words: acyclic retinoid, hepatoma, cell cycle control, nuclear retinoic acid receptor, chemoprevention

ACR AND HEPATOMA

There is increasing interests in the use of vitamins and their related compounds for the treatment and prevention of a variety of diseases including cancer[1,2]. Retinoids are a group of structural and functional analogues of vitamin A that exhibit major effects on cell proliferation and differentiation, and also on pattern formation during development[3]. During the past ten years, there has been increasing evidence that both natural and synthetic retinoids can exert an inhibitory effect on the development of several types of human carcinomas[4,5]. For instance, all-*trans*-retinoic acid (ATRA), 13-*cis*-retinoic acid[1], and *N*-(4-hydroxyphenyl) retinamide (HPR, fenretinide) have been used as therapeutic or chemopreventive agents for head and neck squamous cell carcinoma (HNSCC), esophageal SCC, leukoplakia, breast carcinoma, ovarian carcinoma, and acute promyelocytic leukemia[6-14]. Treatment with 13-*cis*-RA has been demonstrated to reduce the occurrence of second primary carcinomas in patients with previously resected HNSCCs[15]. Clinical trial using 9-*cis*-RA are under way in several types of human solid tumors[16].

In 1981, Muto[17] synthesized a novel retinoid, (2*E*, 4*E*, 6*E*, 10*E*)-3, 7, 11, 15-tetramethyl-2, 4, 6, 10, 14,-hexadecapentaenoic acid, named ACR (Fig. 1), that binds to the cellular retinoic acid binding protein (CRABP). This unique compound inhibited carcinogen-induced hepatocarcinogenesis in rats (18) and spontaneously occurring hepatoma in mice[18]. This agent also inhibited growth and induced apoptosis in the Huh7 human hepatoma cell line[19,20]. In 1996, Muto *et al.* demonstrated that oral administration of ACR (600mg/day) for 12 months significantly reduced the recurrence of primary hepatoma in patients who had their initial lesions resected[21]. Three years later, the follow-up study confirmed the preventive effect of ACR on second primary hepatomas[22]. In these studies, ACR did not cause the typical toxic side effects seen with conventional retinoids[21-23]. These side effects by conventional retinoids include dryness of the skin and lips, skin rash, nasal congestion and, in some cases receiving high doses, a severe "retinoic acid syndrome" or "hypervitaminosis A syndrome"[24,25]. Although the

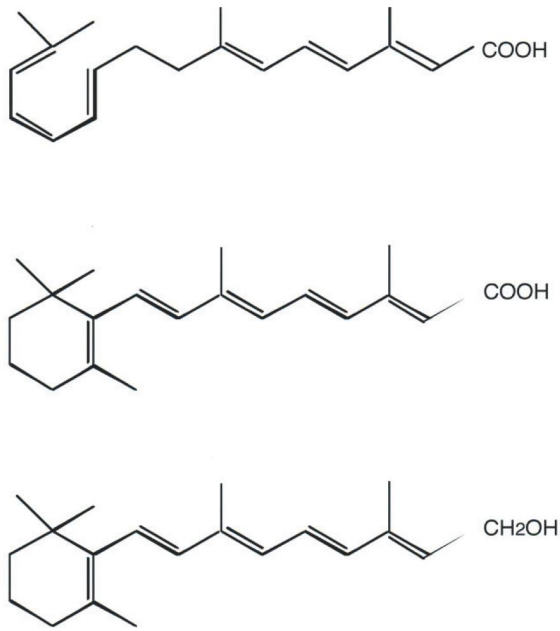

Fig. 1 Chemical structures of (2*E*, 4*E*, 6*E*, 10*E*)-3, 7, 11, 15-tetramethyl-2, 4, 6, 10, 14,-hexadecapentaenoic acid (acyclic retinoid) (A), all-*trans* retinoic acid (B), and vitamin A (C).

above-described results obtained with ACR in experimental and clinical studies are promising, the molecular mechanisms by which this agent exerts tumor suppression are not known.

Hepatomas (hepatocellular carcinomas) are a relatively common malignancy, ranking fifth in frequency (fifth in male and eighth in female) worldwide[26, 27]. Each year around 372,000 new cases are diagnosed and about 427,000 patients died from this disease[28]. Persistent infection with hepatitis B and C viruses accounts for approximately 80% of the cases of hepatoma[27]. Most of the cases of hepatoma occur in developing countries in Eastern Asia and Middle and West Africa[27]. However, the incidence of hepatoma has been rising in western European countries, the United States, and Japan[29, 30]. In Japan and the United States, it is estimated that approximately 1.5 and 3.9 million people are infected with hepatitis C virus and there has been a progressive increase in the number of hepatoma cases over the past two decades[29, 30]. Unfortunately, most of the cases of hepatoma are not curable because extensive resection is not possible, there is severe liver dysfunction caused by cirrhosis, and/or it is difficult to identify the disease at an early stage. Furthermore, at the present time there is no effective chemotherapy for cases with advanced disease.

CELL CYCLE CONTROL MOLECULES

Cyclins play a key role in cell cycle control because of their specific and periodic expression during cell cycle progression. The D-type cyclins (cyclin D1, D2, and D3) complex with cyclin-dependent kinase (cdk)-4 and cdk-6 and thereby regulate transition from the G1 phase into the S phase by phosphorylation and inactivation of pRb[31]. The activities of these cyclin D/cdk complexes are negatively regulated by the cdk inhibitors $p12^{INK4a}$, $p21^{CIP1}$, and $p27^{KIP1}$ [31]. Amplification and/or overexpression of the cyclin D1 (*bcl1, PRAD1,* and *CCND1*) gene has been found in about 13% of human hepatomas[32, 33]. The cyclin D1 protein is also frequently overexpressed in a variety of other human carcinomas[31]. These findings suggest that aberrant expression of cyclin D1 may play a critical role in the development of human carcinomas including hepatoma. In fact, overexpression of cyclin D1 is found to be sufficient to initiate hepatocarcinogenesis in transgenic mice[34]. Thus cyclin D1 can have an oncogenic action in the liver. Therefore this molecule is a potential target for prevention and therapy of hepatoma.

Recent evidence demonstrates that transcription of the cyclin D1 gene is stimulated by activation of the β-catenin/T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) signaling pathway in human colon carcinoma[35, 36]. The adenomatous polyposis coli (APC) protein regulates β-catenin through the ubiquitination pathway after phosphorylation by glycogen synthase kinase-3β (GSK3β)[36]. In human colon carcinoma cells that have mutations in the APC gene or β-catenin gene, b-catenin is accumulated in the nuclei[36]. These findings explain why cyclin D1 is often overexpressed in human colon carcinoma[37, 38]. Mutations in the APC gene have not been detected in either animal or human hepatomas[39, 40]. However, β-catenin is normally targeted for proteolytic degradation by phosphorylation of Ser and/or Thr residues in the N-terminal portion by GSK-3β[41, 42]. Mutations in the β-catenin gene that prevent this phophorylation have been found in a wide variety of human carcinomas, including hepatoma[43-45], and also in carcinogen-induced hepatoma in mice and rats[46-48]. An increased expression of the β-catenin protein has recently been found to be associated with a poor prognosis in patients with hepatoma[49]. However, the precise role of the β-catenin signaling pathway in controlling cyclin D1 expression in

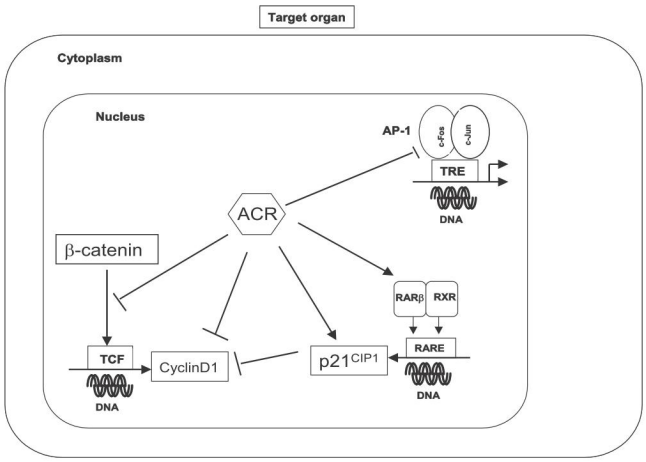

Fig. 2 Primary G1-arresting events caused by ACR are accompanied by a subsequent decline in the levels of the cyclin D1 protein[65, 67]. The decreased levels of expression of the cyclin D1 protein is proceeded by a decline in cyclin D1 mRNA. ACR also inhibits transcription from the cyclin D1 promoter. This demonstration together with an additional finding that the cyclin D1 protein levels are not likely to be regulated by proteasome mediated pathway leads to postulate that the regulation of the cyclin D1 levels in response to ACR is due to the repression of the cyclin D1 promoter ctivity as opposed to ubiquitin-proteasome mediated degradation of the cyclin D1 protein. In HepG2 cells, β-catenin stimulates the transcription of cyclin D1 through the transcription factor TCF. ACR is capable of repressing the activation mediated by β-catenin/TCF however this repression is not specific to any known TCF response element. ACR induces a rapid and dramatic rise in the levels of $p21^{CIP1}$ and RARβ[65, 66]. The promoter region of the $p21^{CIP1}$ gene contains a functional RARE sequence and that this sequence confers retinoid induction of $p21^{CIP1}$ mRNA through RAR-RXR heterodimers[68]. ACR inhibits both AP-1 and c-Fos promoter activity in HepG2 and HCE7 cells[66, 67]. For additional details see text.

human hepatoma cells has not been previously examined.

NUCLEAR RETINOIC ACID RECEPTORS

The activity of various retinoids is considered to be mediated through interactions with two subfamilies of nuclear retinoic acid receptors, RARs and RXRs[50]. These subfamilies include three distinct subtypes, designated α, β, and γ, that are encoded by distinct genes[51]. The nuclear retinoic acid receptors consist of a conserved modular structure, which contains six domains (A-F), from the amino terminus to carboxyl terminus of the molecule[52]. The C region is the DNA binding domain which recognizes and binds to retinoid response elements. The E region is the ligand binding domain and comprises the carboxyl half of the protein. Each RAR gene can generate numerous isoforms (i.e., RAR γ1, γ2, and γ3) which diverge in the amino-terminal region of the protein. RXRs form homodimers or heterodimer with RARs or PPARs[50]. All of these receptors are ligand-dependent transcription factors and control the activity of target genes by binding to DNA response elements, including RAREs (DR5) and RXREs (DR1), located in the promoter regions of the target genes[52]. The RARs bind both ATRA and 9 -*cis*-RA, while the RXRs bind only 9-*cis*-RA[53]. These receptors are also thought to bind a variety of synthetic retinoids, some of which preferentially bind to specific subtypes[4].

In general, normal oral mucosa epithelial cells express RARα, RARβ, RARγ, and RXRα[12, 54, 55], however preneoplastic lesions in the oral mucosa and HNSCCs often represent selective suppression of RARβ expression[12, 56, 57]. These findings suggest that loss of RARβ expression is associated with the development of HNSCCs. In addition, ATRA induces increased expression of the RARβ protein in immortalized human bronchial epithelial cells and in human HNSCC cell lines[11, 58-60]. Furthermore treatment of patients with premalignant oral lesions, metaplastic bronchial epithelium, or renal cell carcinoma with 9 - or 13-*cis* RA can induce increased expression of RARβ in these lesions[61-63]. Previous studies demonstrated that treatment of the human hepatoma cell line with ATRA also causes a marked increase in the level of RARβ mRNA but not the level of RARα mRNA[64]. However, HPR selectively activates transcription of RARγ and to a lesser extent RARβ, but not transcription of RARα and RXRα in human breast carcinoma cell lines[27]. Novel synthetic retinoids that bind selectively to RXRs are termed rexinoids[5]. These compounds have been found to be potent chemopreventive agents in experimental animal systems[5]. In fact, LGD1069 (targretin or bexarotene) and LG100268, a prototype of rexinoids, are highly effective in rat mammary carcinogenesis prevention model systems[5]. LGD1069 also inhibits proliferation of human HNSCC cell lines[28]. These findings suggest that different retinoids can act through different nuclear retinoic acid receptors

to alter the expression of genes that inhibit the growth of cancer cells.

GROWTH INHIBITION OF HUMAN HEPATOMA CELLS BY ACR

In a recent study[65], we found that ACR inhibited the growth of three human hepatoma cell lines, HepG2, Hep3B, and Huh7. In HepG2 cells this inhibition was associated with an arrest of the cell cycle in G0/G1, increased cellular levels of $p21^{CIP1}$, decreased levels of ppRb, and decreased levels of cyclin D1, but no significant changes in the levels of the $p16^{INK4a}$, $p27^{KIP1}$, cdk4, cdk6, GSK-3β, and β-catenin proteins (Fig. 2). ACR also caused a decrease in the level of cyclin D1 mRNA. Co-treatment of HepG2 human hepatoma cells with the proteasome inhibitor LLnL did not prevent the ACR-induced decrease in the cyclin D1 protein, in contrast to the protective effect of LLnL on the cyclin D1 protein in cells treated with ATRA. Furthermore, in transient transfection reporter assays, ACR, but not ATRA, inhibited transcription from the cyclin D1 promoter. We also found that in hepatoma cells, as described in human colon carcinoma cells, cyclin D1 promoter activity is markedly stimulated by the β-catenin/TCF pathway. Even in the presence of excess β-catenin ACR markedly inhibited the transcriptional activity of the cyclin D1 promoter but also exerted other inhibitory effects on cyclin D1 transcription. The above-described demonstration showing that ACR inhibits the expression of cyclin D1 and exerts other inhibitory effects in human hepatoma cells should further encourage the use of this novel drug in the chemoprevention and treatment of hepatoma and other types of human carcinomas.

ACR ACTIVATES RETINOIC ACID RECEPTOR β AND INDUCES TRANSCRIPTIONAL ACTIVATION OF $p21^{CIP1}$

In a recent study[66], we further investigated the molecular effects of ACR on the HepG2 human hepatoma cell line, focusing on the expression of nuclear retinoid receptors and the cell cycle inhibitor protein $p21^{CIP1}$ (Fig. 2). RT-PCR assays and western blot analyses indicated that these cells express RARα, RARβ, RARγ, RXRα, RXRβ and PPARγ mRNAs. Treatment with ACR caused a rapid induction, within 3 h, of RARβ mRNA and the related protein but there was no significant change in the levels of the mRNAs or proteins for RARα and RARγ, RXRα, RXRβ and PPARγ. There was also a rapid increase in $p21^{CIP1}$ mRNA and protein in HepG2 cells treated with ACR and this induction occurred via a p53-independent mechanism. In transient transfection reporter assays, we co-transfected the retinoic acid response element (RARE)-CAT reporter gene into HepG2 cells together with an RARβ expression vector. RARβ expression markedly stimulated CAT activity (up to about 4-fold) after the addition of ACR. However, CAT activity in the presence of ACR was only about 2-fold higher than that in the absence of ACR, when cells were co-transfected with either RARα, RARγ, or RXRα. These findings suggest that the growth inhibitory effects of ACR are mediated, at least in part, through RARβ and suggest that both RARβ and $p21^{CIP1}$ play critical roles in the molecular mechanisms of growth inhibition induced by ACR. We also found similar effects of ACR in a human squamous cell carcinoma cell line[67]. A recent study demonstrates that the promoter region of the $p21^{CIP1}$ gene contains a functional RARE sequence and that this sequence confers retinoid induction of $p21^{CIP1}$ mRNA through RAR-RXR heterodimers[68]. Therefore, the $p21^{CIP1}$ gene appears to be a retinoid target gene[68]. In addtion, $p21^{CIP1}$ has been shown to be a primary response gene in the induction of differentiation of HL-60 human leukemia cells by ATRA[69]. AP-1 is composed of the apparent ability proto-oncogenes c-Jun and c-Fos and its activity is often associated with cell proliferation and tumor progression[70]. RARβ displays a strong ATRA-independent inhibition of AP-1 activity, whereas inhibition of AP-1 activity by RARα and RARγ is ARTA dependent[71]. RARα also inhibits AP-1 activity by directly binding to the c-Jun complex[72]. In the HepG2 human hepatoma and the HCE7 human squamous cell carcinoma cell lines we found that ACR inhibits both AP-1 and c-Fos promoter activity in a dose dependent fashion[68, 69]. Thus the growth inhibitory effects by ACR might also occur through inhibition of AP-1 and/or c-Fos activity.

CLINICAL APPLICATION OF ACR

In clinical studies in which ACR was administered to patients at a dose of 600 mg/day the serum

level was about 0.2 μM[23], while in patients who received 300-400 mg/day of ATRA the ATRA serum concentration was only about 0.15 μM[1]. In cell culture studies conventional retinoids have been used in the range of 1-10 μM to inhibit cell proliferation, depending on the cell line and growth conditions[1,60,73-76]; most of our recent studies were done with 30 μM ACR[65-67]. Additional studies are required to determine whether the molecular effects of ACR that have been seen in hepatoma cell cultures also occur in tumors *in vivo*. Despite its unusual chemical structure, ACR's molecular mechanisms of action resemble those of several retinoids. A serious limitation in the clinical use of ATRA and other retinoids in cancer chemoprevention and therapy is their adverse effects, particularly with prolonged administration. These side effects include a severe "retinoic acid syndrome"[24,25]. On the other hand, thus far clinical studies with ACR have not revealed the adverse effects seen with conventional retinoids[21,22]. The reasons why ACR appears to be better tolerated are not known, and this aspect warrants further study. A phase I study has already been conducted to determine the maximum tolerated dose of ACR and to identify the types of possible adverse effects caused by this compound. A phase II study in a wide scale objecting second primary hepatoma cases will start this year. In the near future this novel drug may provide a valuable treatment option for patients with advanced disease.

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