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Chemoprevention of hepatocellular carcinoma by acyclic retinoid

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1. ABSTRACT

The prognosis for patients with hepatocellular carcinoma (HCC) is poor and effective prevention strategies are urgently required. Here, we review abnormalities in the expression and function of retinoids and their receptors, and how they play a critical role in the development of HCC. In particular, a malfunction of RXRα due to phosphorylation by Ras-MAPK signaling pathway is profoundly associated with liver carcinogenesis and thus may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), a synthetic retinoid, inhibits Ras-MAPK activation and RXRα phosphorylation, thereby suppressing growth in HCC-derived cells. In clinical trials, ACR has been shown to improve patient survival by preventing viral HCC development, a possible manifestation of the concept of "clonal deletion" therapy. "Combination chemoprevention" with ACR as the key drug has great potential to become an effective strategy for the prevention of liver carcinogenesis. In summary, both basic and clinical research strongly suggest that ACR plays a critical role in preventing the development of HCC and that "clonal deletion" therapy is one of the most practical approaches for this purpose.

2. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for 500,000 to 600,000 deaths per year. The development of HCC is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). This fact indicates that HCC is a major health problem in Eastern as well as Western countries where hepatitis viral infection is endemic, and the incidence is increasing (1-3). However, in spite of strenuous efforts to develop effective methods of diagnosis and treatment, there has been limited improvement in the prognosis for this malignancy. A major obstacle for HCC therapy is the high frequency of tumor recurrence after curative treatment; the recurrence rate at 5 years after definitive therapy may exceed 70% (4, 5). At present, there are no effective chemotherapeutic agents for this malignancy. Therefore, there is a critical need to develop more effective strategies for the chemoprevention and chemotherapy of HCC to improve the prognosis for patients with this malignancy; for this purpose, we must elucidate the molecular mechanisms underlying hepatocarcinogenesis. Among the several causal factors for the development of HCC,

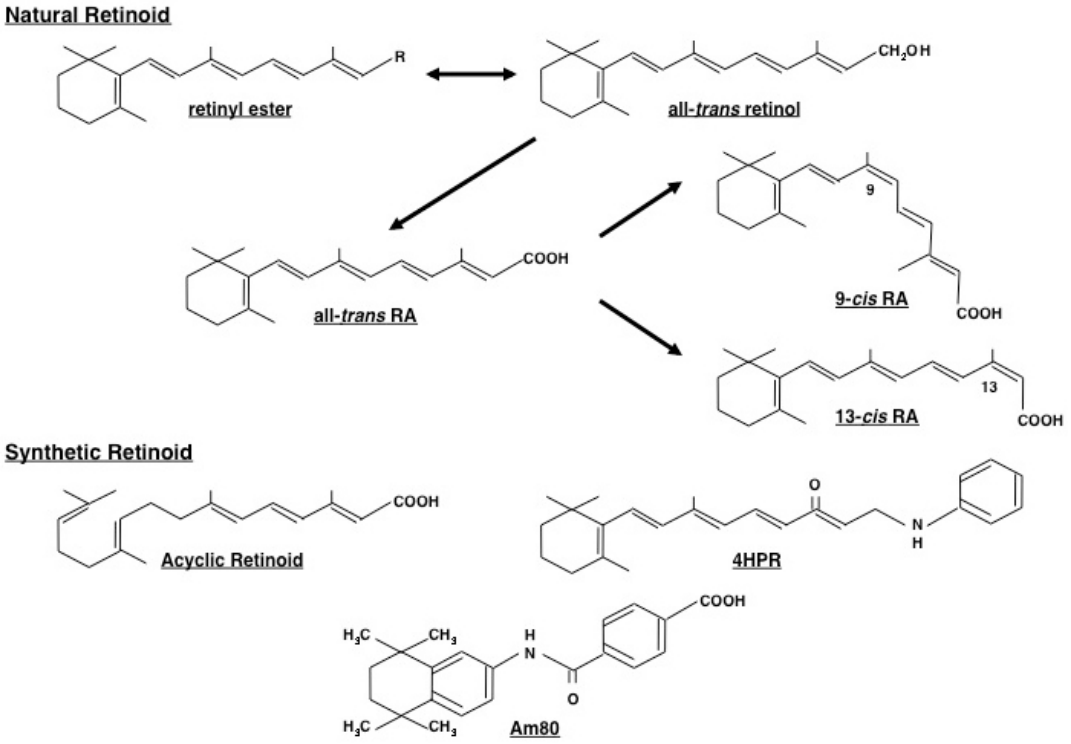

Figure 1. Chemical structures of natural and representative synthetic retinoids. Retinyl esters (mainly retinyl palmitate, R: fatty acid), stored in the liver stellate cells, are hydrolyzed to retinol. Retinoic acid (RA) is biosynthesized from retinol via the intermediate metabolite retinal by oxidization in the cells of peripheral tissues. Three well-known isomers of RA, all-*trans* RA, 9-*cis* RA, and 13-*cis* RA activate retinoid receptor, RARs, whereas only 9-*cis* RA activates the other receptor, RXRs. All-*trans* RA inhibits proliferation and induces granulocytic differentiation in leukemic cells of acute promyelocytic leukemia and thus is a first-line drug for this disease. A number of synthetic retinoids have been developed for pharmacological applications including cancer chemoprevention. ACR and N-(4-hydroxyphenyl) retinamide (4HPR) successfully prevented the development of HCC and breast cancer, respectively, in clinical trials. Am80 (Tamibarotene) is approved for relapsed or refractory acute promyelocytic leukemia in Japan.

phosphorylation of retinoid X receptor-α (RXRα) by the Ras-MAPK signaling pathway is considered to play a key role (6-9).

Because of the high incidence of recurrence and the development of secondary tumors (4, 5), the curative treatment for HCC is difficult once this malignancy has developed. The high risk group, including patients infected with hepatitis, are easily identified, however. Therefore, cancer chemoprevention, an approach wherein a natural or synthetic chemical compound works to arrest or reverse premalignancies via physiological pathways (10), is one of the most promising strategies for the treatment of HCC, particularly hepatitis virus-positive patients. We previously reported that, in clinical trials, the administration of acyclic retinoid (ACR), a novel synthetic retinoid which targets phosphorylated RXRα (11-13), reduced the incidence of post-therapeutic HCC recurrence and improved patient survival (14-17). In this article, we review evidence that a malfunction of RXRα due to phosphorylation is closely involved in liver carcinogenesis. We also show the pleiotropic effects of ACR in the inhibition of HCC and suppression of cancer growth, especially focusing on the inhibition of RXRα phosphorylation and induction of RARβ and $p21^{CIP1}$ expression. In addition, the possibility of "combination chemoprevention", which uses ACR as a key drug, and the concept of "clonal deletion" therapy, a practical approach to preventing HCC development, are also discussed.

3. RETINOIDS AND THEIR RECEPTORS

Vitamin A and its functional analogues, collectively termed retinoids, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development (18, 19). Retinoids consist of several molecular species, including retinoic acid (RA, an active metabolite that binds to its nuclear receptor), retinol (a transport form in the plasma), and retinylesters (a storage form in the tissues). In addition, large numbers of synthetic retinoids, including ACR, have been developed (Figure 1). Retinoids exert their biological functions primarily by regulating gene expression through 2 distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs, which are both composed of 3 subtypes (α, β, and γ) that are characterized by a modular domain structure. Nuclear retinoid receptors are ligand-dependent transcription

factors; after ligand binding, RXRs form a homodimers, as well as heterodimers with RARs, which interact with the retinoid X response element (RXRE) or the retinoic acid receptor responsive element (RARE) located in the promoter region of target genes, thereby modulating gene expression (18, 19). In addition to RARs, RXRs also form heterodimers with other nuclear receptors including peroxisome proliferator-activated receptors (PPARs), which control energy homeostasis by modulating glucose and lipid metabolism and transport (20). Therefore, RXRs play a fundamental role in controlling normal cell proliferation and metabolism, and act as master regulators of nuclear receptors (19). These facts suggest that retinoid receptors, especially RXRs, are exciting pharmacological targets for the therapies of various human diseases, including cancer and metabolic disease (21, 22).

4. ABNORMALITIES IN THE RETINOID/RETINOID RECEPTOR AXIS AND HCC

Because retinoids and their receptors play an essential role in normal cell proliferation, differentiation, and death (regulation of apoptosis), abnormalities in the expression and function of these molecules, especially RXRα and RARβ, are strongly associated with the development of various human malignancies including HCC. For instance, the *RARβ* gene is an HBV integration site and its expression is markedly decreased in human HCC (23, 24). In the chemical-induced rat liver carcinogenesis model, both RARβ protein and mRNA levels are also decreased in HCC (25). These findings are interesting because among the retinoid receptors, RARβ is thought to be one of the most important receptors in the regulation of cell growth and apoptosis (26).

The expression of RXRα is also decreased not only in HCC and liver cell adenoma, but also in glutathione *S*-transferase placental form-positive foci, a precancerous HCC lesion in the chemical hepatocarcinogenesis model in rats (25). These findings suggest that the repression of RXRα occurs even in the early stage of liver carcinogenesis. Moreover, recent studies have revealed that liver carcinogenesis is accompanied by an accumulation of the phosphorylated (*i.e.* inactivated) form of RXRα (p-RXRα) (27). Specifically, RXRα protein is anomalously phosphorylated at serine and threonine residues, and accumulates in both human HCC tissue and HCC cell lines (9). Phosphorylation at serine 260 of RXRα, a MAPK consensus site, is closely associated with its retarded degradation, low transcriptional activity, and the promotion of cancer cell growth; the abrogation of phosphorylation by a MAPK inhibitor restores the degradation of RXRα in a ligand-dependent manner (9, 11). In addition, although RXRα is unphosphorylated and highly ubiquitinated in a normal liver, rendering it sensitive to proteasome-mediated degradation, p-RXRα is resistant to ubiquitination and proteasome-mediated degradation in both human HCC tissues and a human HCC cell line (28). Furthermore, the phosphorylation of RXRα abolishes its ability to form heterodimers with RARβ, and this is associated with uncontrolled cell growth and resistance to retinoids (29). These findings suggest that the accumulation of p-RXRα, (*i.e.*, non-functional RXRα) may interfere with the function of normal RXRα in a dominant-negative manner, thereby playing a critical role in the development of HCC (Figure 2). There are also some reports that show the analogous effects of phosphorylated RXRα in the negative modulation of its heterodimeric binding partners (30-32). Therefore, the inhibition of RXRα phosphorylation and the restoration of its heterodimeric activity with other nuclear receptors may be an effective and important strategy for the prevention and treatment of certain types of human diseases, especially malignant disorders including HCC (6-8, 33-35).

5. ACR IN HCC CHEMOPREVENTION: EXPERIMENTAL STUDIES

ACR, which was initially developed as an agonist for both RXR and RAR (36, 37), has been demonstrated to produce several beneficial effects on the prevention of HCC development and inhibition of growth in HCC cells (ACR is the same substance as NIK-333 and Peretinoin; Kowa Pharmaceutical Co., Tokyo, Japan; See Figure 3). In rodent studies, ACR inhibits both chemical-induced hepatocarcinogenesis in rats and spontaneously occurring HCC in mice (38). ACR also inhibits growth of HCC-derived cells by inducing cell proliferation and apoptosis, which effects seem to be associated with upregulation of RARβ expression (13, 36, 39-44). In human HCC and squamous carcinoma cells, ACR causes cell cycle arrest in G_0-G_1, increased cellular levels of $p21^{CIP1}$, and decreased levels of cyclin D1 and the phosphorylated form of retinoblastoma proteins (44-46). These findings suggest that RARβ and $p21^{CIP1}$ are one of the critical targets of ACR with respect to growth inhibition and apoptotic induction in cancer cells.

Recent *in vivo* and *in vitro* studies have indicated that ACR not only binds to RXR and RAR, but also reduces the development of HCC and inhibits cancer growth by targeting growth factors and their corresponding receptor tyrosine kinases (RTKs), which play a critical role in activation of the Ras-MAPK signaling pathway (41, 46-50). These reports are significant because the activated Ras-MAPK pathway phosphorylates RXRα, thus contributing to the development of HCC (9, 27). In addition, ACR also restores RXRα function by inactivating the Ras-MAPK signaling system, leading to the dephosphorylation of RXRα, although 9-*cis* RA failed to suppress ERK and RXRα phosphorylation (11). Therefore, ACR, which targets the RTK-Ras-MAPK signaling pathway and RXRα phosphorylation, is a promising agent for the chemoprevention of HCC. The role of RXRα phosphorylation in liver carcinogenesis and its inhibition by ACR are schematically represented in Figure 2.

6. ACR IN HCC CHEMOPREVENTION: CLINICAL STUDIES

An early phase randomized, controlled clinical trial tested the chemopreventive effect of ACR on

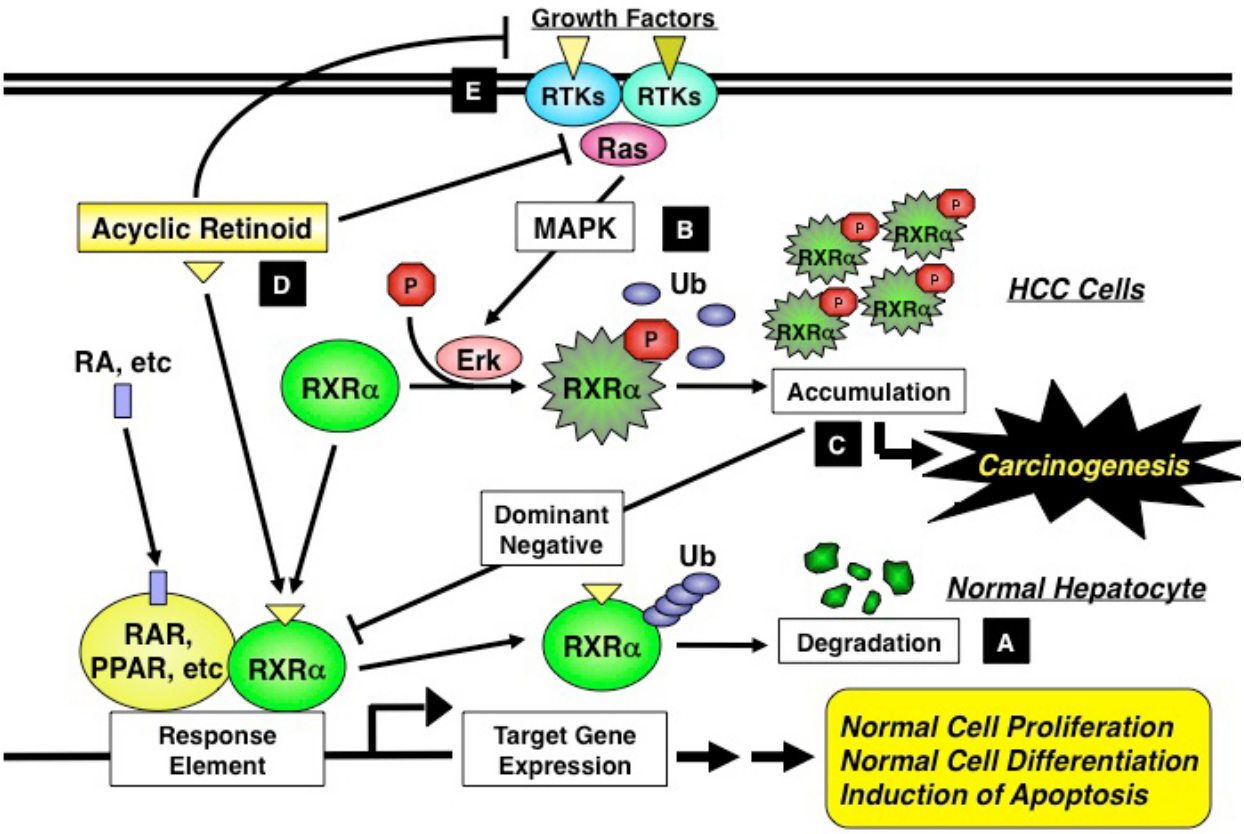

Figure 2. Retinoid-refractoriness due to phosphorylation of RXRα and its restoration by ACR in liver carcinogenesis. In normal hepatocytes, when ACR binds to and activates RXRα, it forms homo- and/or heterodimers with other nuclear receptors including RARs and PPARs, and then activates the expression of target genes that regulate normal cell proliferation and differentiation by binding to the specific response element. Thereafter, RXRα is rapidly ubiquitinated (Ub) and degraded via the proteasome pathway (A). In HCC cells, the Ras-MAPK pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor (B). Furthermore, non-functional phosphorylated RXRα (p-RXRα) is sequestered from ubiquitin/proteasome-mediated degradation, and accumulates in liver cells, interfering with the physiological function of the remaining unphosphorylated RXRα in a dominant negative manner, thereby playing a critical role in liver carcinogenesis (C). ACR is not only a ligand for RXRα but also suppresses the Ras-MAPK signaling pathway, inhibiting RXRα phosphorylation, restoring the function of the receptor, and thus activating the transcriptional activity of the responsive element (D). ACR also directly or indirectly inhibits the ligand (growth factors)-dependent RTK activities (E), which also contributes to the inhibition of Erk and RXRα phosphorylation and suppression of growth in HCC cells.

secondary HCC in patients who received anti-cancer treatment for an initial HCC (14-16). In this trial, oral administration of ACR (600 mg per day) for 12 months significantly reduced the incidence of secondary HCC after a median follow-up period of 38 months (P = 0.04) (14), and improved both incidence (P = 0.002) and survival (P = 0.04) after a median follow-up period of 62 months (15). Relative risk of the development of secondary HCC and death were 0.31 (95% confidence interval, 0.12 to 0.78) and 0.33 (0.11 to 0.79), respectively (14, 15). Moreover, the preventive effects of ACR lasted up to 199 weeks after randomization or 151 weeks after completion of ACR administration (16).

A phase II/III trial of ACR confirmed its effectiveness in preventing secondary HCC in hepatitis C virus-positive patients in a multicenter, large-scale (n = 401) randomized placebo-controlled trial; oral administration of 600 mg of ACR per day was tolerated and had a strong effect on the prevention of secondary HCC with a hazard ratio of 0.27 (0.07 to 0.96) after 2 years (17). The results of these clinical trials suggest that ACR is a novel first-line therapy to reduce the development of secondary HCC.

7. "CLONAL DELETION" THERAPY FOR HCC

Liver carcinogenesis is characteristically multicentric in nature, a phenomenon which is expressed by the term "field cancerization" (51). The poor prognosis for HCC, which is associated with a high incidence of recurrence and development of secondary tumors, is

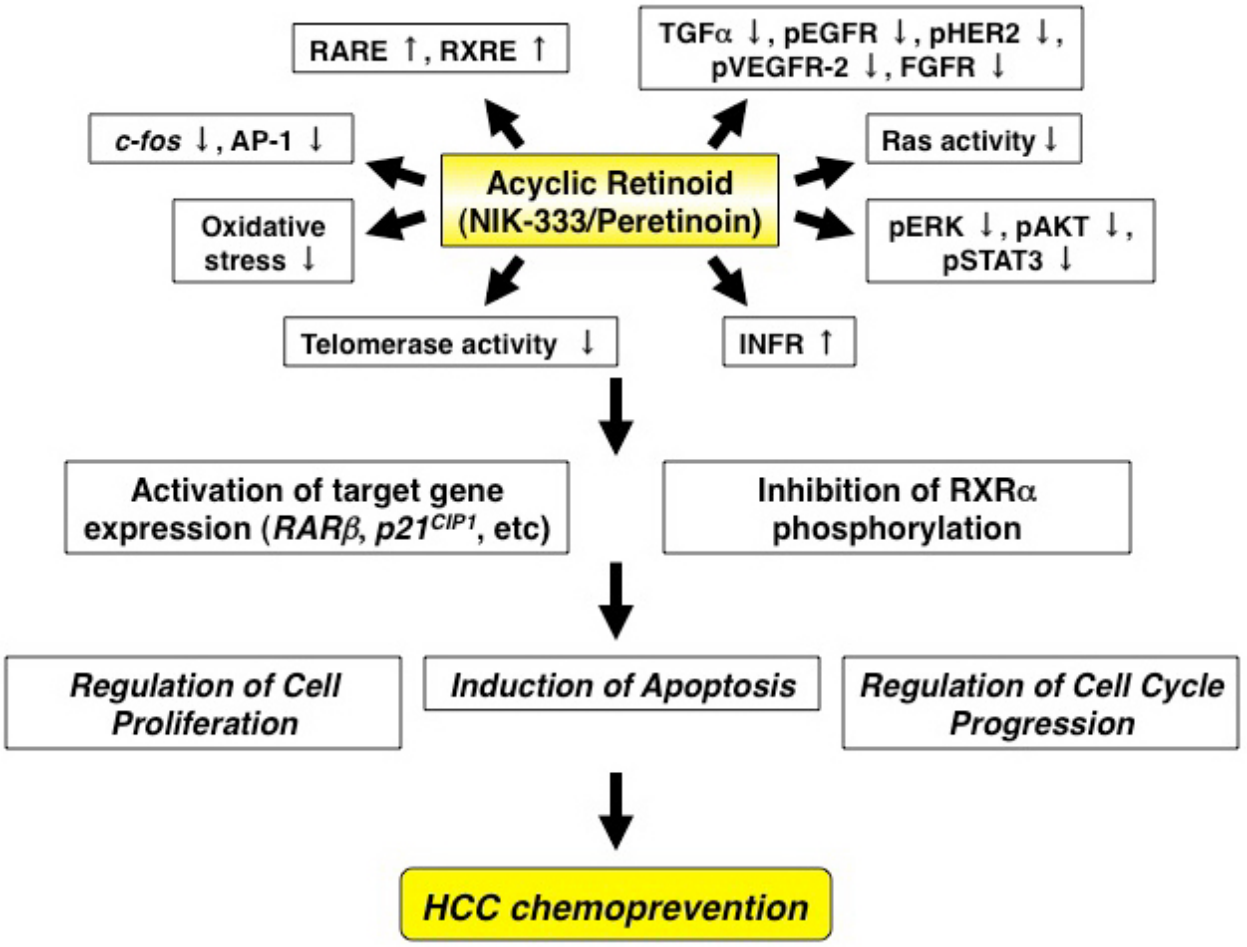

Figure 3. Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes, such as *RARβ* and $p21^{CIP1}$, by upregulating the promoter activity of RARE and RXRE. In addition, ACR suppresses cancer cell growth by inhibiting activation and expression of some types of RTKs, including EGFR, HER2, VEGFR-2, and FGFR, which contribute to the subsequent inhibition of Ras-MAPK activation and RXRα phosphorylation. Phosphorylation of Akt and Stat3 proteins are also inhibited by ACR. Induction of RARβ and restoration of the function of RXRα due to dephosphorylation by ACR leads to cooperative regulation of cell proliferation, cell cycle progression, and induction of apoptosis, thus preventing the development of HCC. ACR also induces the expression of IFN receptor (INFR), inhibits transcriptional activity of c-*fos* and AP-1 promoters, and down-regulates telomerase activity in HCC and squamous cell carcinoma cells. ACR also suppresses liver tumorigenesis by repressing oxidative stress. Detailed discussion of these findings may be found in previous articles (6-8, 11-13, 36-50, 53, 58, 60-62).

particularly relevant to field cancerization. Once a liver is exposed to continuous carcinogenic insults, such as hepatitis viral infection and alcohol toxicity, the whole exposed liver is regarded as a precancerous lesion which possesses multiple as well as independent premalignant or latent malignant clones. Hence, even if the first cancer is diagnosed and removed early, the next clone essentially arises to form a secondary HCC. Therefore, the most effective strategy for HCC chemoprevention is the deletion of latent malignant clones (clonal deletion) and inhibition of the evolution of such clones (clonal inhibition) before they expand into clinically detectable tumors. We have proposed that implementation of this novel concept, "clonal deletion" therapy, which is defined as the removal of latent malignant (or premalignant) clones that are invisible by diagnostic imaging from the liver when it is in a hypercarcinogenic state, is fundamental to the chemoprevention of HCC (Figure 4) (6-8).

ACR has been used to effectively demonstrate this concept in the clinical setting. In the clinical trial, serum levels of lectin-reactive α-fetoprotein factor 3 (AFP-L3), which indicates the presence of latent (*i.e.*, invisible) malignant clones in the remnant liver, were significantly reduced by 12-month administration of ACR (52). This observation indicates that ACR eliminates or removes the AFP-L3 producing premalignant clones from the remnant liver before they expanded into clinically detectable (*i.e.*, visible) tumors, thereby inhibiting secondary HCC. Moreover, ACR suppressed the appearance of serum AFP-

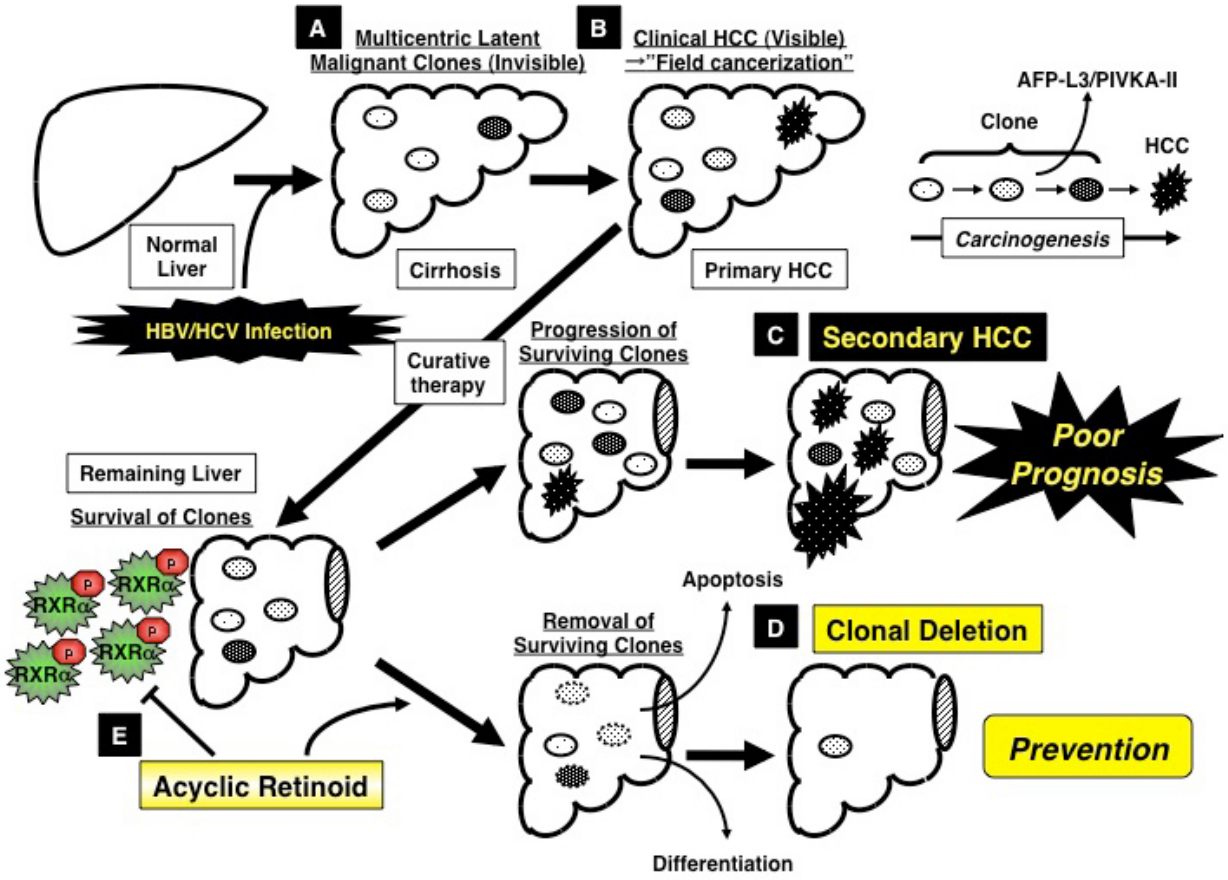

Figure 4. The concept of "clonal deletion" therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a "precancerous field", which consists of multiple latent malignant clones that arise through multicentric carcinogenesis and are clinically undetectable by image analysis (invisible) (A). These multiple clones demonstrate different grades of malignancy in the cirrhotic liver and, at some point, turn into clinical (visible) HCC ("field cancerization") (B). Even when primary HCC is found and removed early, the other clones survive in the remaining liver and grow into secondary HCC, which is a major cause of the poor prognosis for patients with this malignancy (C). Therefore, one of the most promising strategies to prevent secondary HCC is deletion of such transformed clones by inducing cell differentiation or apoptosis before they expand into clinically detectable tumors (the concept of "clonal deletion" therapy) (D). ACR, which targets phosphorylated RXRα (E), prevents the recurrence and development of secondary HCC via the mechanism described by this concept; ACR decreased the serum levels of AFP-L3 and PIVKA-II, which are produced by latent malignant clones, thus demonstrating the eradication and inhibition of these clones. Once such clones are deleted, the preventive effect on HCC lasts several years without continuous administration of ACR. Therefore, ACR can significantly improve the survival rate of such patients.

L3 in patients whose AFP-L3 levels were negative at trial enrollment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group; these patients had a significantly higher risk of secondary HCC (52). This finding suggests that, in addition to elimination, ACR actively inhibits the development of AFP-L3-producing clones, which have the potential to become HCC. This is one of the reasons why only a short-term administration (12 months) of ACR exerted a long-term preventive effect on HCC development for several years after termination of treatment (16). It takes several years for the next cancer clones to arise clinically once they are eliminated or inhibited. Therefore, the promise of clonal deletion seems to be therapeutic rather than preventive, and ACR prevents the development of HCC by this mechanism.

8. "COMBINATION CHEMOPREVENTION" OF HCC USING ACR AS THE KEY DRUG

Combination therapy is often advantageous because it provides the potential for synergistic effects between specific drugs; ACR is no exception in this regard. For instance, ACR acts synergistically with interferon (IFN)-β in suppressing growth and inducing apoptosis in human HCC cell lines via upregulation of type 1 IFN receptor and Stat1 expression by ACR (53). The combination of ACR plus vitamin K_2 (VK_2) synergistically inhibits cell growth and induces apoptosis in HCC cells

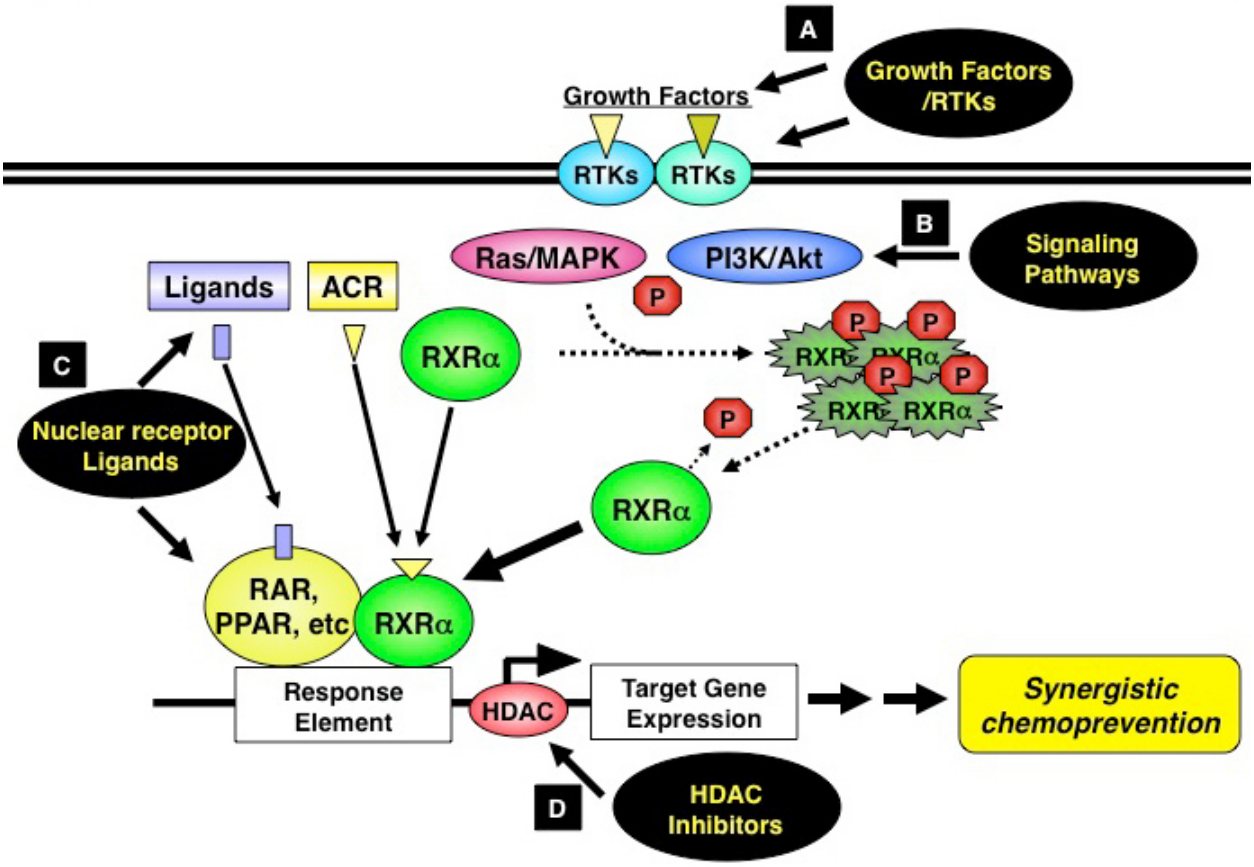

Figure 5. The possibility of "combination chemoprevention" for HCC using ACR as the key agent. Dephosphorylation of RXRα and subsequent restoration of the function of this nuclear receptor are critical to prevent the development of HCC. Therefore, the agents which target growth factor and their corresponding RTKs (A), as well as their related signaling pathways (B), including the Ras-MAPK and PI3K-Akt signaling pathways that phosphorylate RXRα, might be good partners for ACR to exert synergistic effects on the chemoprevention of HCC. The ligands for the nuclear receptors, which form heterodimers with RXR such as RAR and PPAR (C), are also able to enhance the chemopreventive effect of ACR through the activation of target gene expression. HDAC inhibitors increase the expression of ACR-target genes by remodeling the chromatin template and increasing histone acetylation, which suggests that the combination of ACR plus HDAC inhibitors may also be a promising regimen for HCC chemoprevention (D).

without affecting the growth of normal human hepatocytes (12). These findings are significant when considering the clinical use of ACR because both IFN and VK_2 are expected to exert preventive effects on the development and recurrence of HCC (54-57). Therefore, we assume that "combination chemoprevention" using ACR as the key agent may be a useful strategy to prevent the development of HCC.

The expected mechanisms of ACR-based combination chemoprevention are schematically summarized in Figure 5. Initially, specific agents that target the Ras-MAPK signaling pathway and its upstream RTKs are among the most promising partners for ACR because these agents dephosphorylate RXRα. Indeed, ACR and VK_2 cooperatively inhibit activation of the Ras-MAPK signaling pathway, thus suppressing the phosphorylation of RXRα and the growth of HCC cells (12). The combination of 9-*cis* RA (58) or ACR (unpublished data) plus trastuzumab, a humanized anti-human epidermal growth factor receptor-2 (HER2) monoclonal antibody, synergistically inhibits growth and induces apoptosis in HCC cells via cooperative inhibition of the activation of HER2 and its downstream signaling molecules, including ERK and Akt, and subsequent dephosphorylation of RXRα. Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, acts synergistically to induce apoptosis and G_0-G_1 cell cycle arrest in HCC cells by inhibiting phosphorylation of RXRα, ERK, Akt, and GSK-3β proteins (13).

In addition to dephosphorylation of RXRα, induction of nuclear receptors that dimerize RXR, such as RAR and PPAR (33, 59), and recruitment of their ligands may also exert synergistic growth inhibition in cancer cells when combined with ACR. Both valproic acid (13) and OSI-461 (43), a potent derivative of sulindac sulfone, enhance the ability of ACR to raise the cellular levels of

RARβ and $p21^{CIP1}$, thereby markedly increasing the RARE and RXRE promoter activities and inducing apoptosis in HCC cells. Therefore, these combinations may also be an effective regimen for the chemoprevention and chemotherapy of HCC.

9. PERSPECTIVE

The prevention of HCC is an urgent task on a global scale, and one of the most practical approaches to the accomplishment of this purpose is "clonal deletion" therapy. Experimental studies strongly suggest that RXRα phosphorylation is profoundly involved in liver carcinogenesis and thus may be a critical target for HCC chemoprevention. Clinical trials reveal that ACR, which inhibits RXRα phosphorylation but induces RARβ expression, is a promising candidate for HCC chemoprevention by putting the concept of "clonal deletion" in practice. ACR-based combination chemoprevention, which is expected to exert synergism, also holds great promise as a master therapeutic for HCC chemoprevention. In conclusion, ACR may play a critical role in preventing HCC development when it is used alone or combined with other drugs and, therefore, early clinical application of this agent is greatly anticipated.

10. ACKNOWLEDGEMENTS

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Abbreviations: ACR, acyclic retinoid; AFP-L3, lectin-reactive α-fetoprotein factor 3; HBV, hepatitis B virus; HCC, Hepatocellular carcinoma; HCV, hepatitis C virus; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor-2; IFN, interferon; MAPK, mitogen-activated protein kinase; PIVKA-II, protein induced by vitamin K absence or antagonist-II; PPAR, peroxisome proliferator-activated receptors; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid receptor responsive element; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; RXRE, retinoid X response element; VK_2, vitamin K_2

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